The effects of diffuse pollution on the European eel *Anguilla anguilla* (Linnaeus, 1758)

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Declaration

Whilst registered as a candidate for the degree of Doctor of Philosophy, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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Abstract

In the last 40 years, the population of European eel (*Anguilla anguilla L.*) has declined dramatically and is now considered to be outside safe biological limits. In 2007 the European Union implemented an "Eel Recovery Plan" regulation, in order to assist in the recovery of the species back to the previous sustainable levels. The major factors regulating eel populations are still unknown and until there is an understanding of the factors causing the low recruitment of eels, the success of any management plans and conservation measures may be limited. One factor considered important in regulating the eel population is pollution.

The major aim of these studies was to investigate the impact of environmental contaminants on eel throughout their life cycle. Laboratory and field studies were carried out to assess the impact of environmental levels of pesticides (atrazine, fenitrothion, pendimethalin, chlortoluron, flusilazole, copper oxychloride, metaldehyde and chlorpyrifos), metals (copper, lead, zinc and chromium) and flame retardants (tributyl phosphate and hexabromocyclododecane) on the transition of juvenile and adult eels between freshwater and the marine environment and growth and feeding during freshwater residency.

Exposure to tributyl phosphate (TBP) in freshwater had some effect on physiological (plasma levels of glucose, sodium and chloride and kidney Na^+/K^+ ATPase) parameters associated with the silvering process in the eel but not on the morphology or the migratory behaviour during the transition from freshwater to the marine environment. Exposure to a mixture of pesticides (pendimethalin, chlortoluron, flusilazole, copper oxychloride, metaldehyde and chlorpyrifos) did modify the migratory behaviour of eels during the early stages of the freshwater migration but did not have any effects on the physiology of saltwater adaptation. Exposure to atrazine did not impair the olfactory system of eels and they were able to detect compounds released by prey items. Exposure of juvenile (glass eels) to 0.5 μg l^{-1} of tributyl phosphate did not have an impact on their freshwater adaptation as they were able to survive the movement between salt and freshwater. Glass eels exposed to a range of low concentration of metals (copper, lead, zinc and chromium) all demonstrated

significant damage to their DNA. The long term impact of DNA damage is not known or whether this would reduce survival in the eels.

The results of the study indicate that exposure to contaminants as the eels migrate between the freshwater and marine environments has probably only a minor role in regulating the eel population.

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%	Percentage
±	plus or minus
°E	degrees East
ºC	degree Centigrade
°N	degrees North
°W	degrees West
µg	Micrograms
µg g^{-1}	microgram per gram
µg kg^{-1}	microgram per kilo
µg l^{-1}	microgram per litre
µl	Microliter
µM	Micromolar
‰	parts per thousands
A	Adenine
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AChE	Acetylcholinesterase
Ag^{+}	Silver
Ag–AgCl	Silver-silver chloride
ALS	acoustic listening stations
C	Cytosine
Ca^{2+}	Calcium ion
Cd	Cadmium
Cd^{2+}	Cadmium ion
CF	condition factor
ChE	Cholinestesterase
Cl^{-}	Chloride ion
cm	Centimetre
CO_2	Carbon Dioxide
Cr	Chromium

Cu	Copper
Cu^{+}	Copper ion
CYP1A	cytochrome P4501A
d.f.	degrees of freedom
DMBA	7,12-Dimethylbenz[a]anthracene
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
e.g.	exempli gratia
EA	Environment Agency
EDTA	Ethylenediaminetetraacetic acid
EF-1	elongating factor 1
EGCs	emerging organic groundwater contaminants
EI	eye index
EMP	Eel Management Plan
EOG	electro-olfactogram
EPA	US Environmental Protection Agency
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FW	Freshwater
g	Gram
g	Centrifugal force
G	Guanine
$g\ l^{-1}$	grams per litre
GSI	gonadosomatic index
H^{+}	Hydrogen ion
HBCD	Hexabromocyclododecane
HCl	Hydrochloric acid
HCO_3^{-}	Hydrogen carbonate
hr	Hour
HSI	hepatosomatic index
HW	high water
ICES	Internationa Council for the Exploration of the Sea
IHN	infectious haematopoietic necrosis

IPN	infectious pancreatic necrosis
K^+	Potassium ion
KCl	Potassium Chloride
kHz	Kilohertz
km	kilometers
km day^{-1}	kilometers per day
km^2	square kilometres
l	Litre
l min^{-1}	litre per minute
L13	Ribosomal protein L13a
LC_{50}	Lethal concentration for 50% of tested individuals
LD_{50}	lethal dose for 50% of the tested animals
LMP	Low melting point
LW	low water
m	Metre
M	Molar
m sec^{-1}	metre per second
mA	milliAmpere
mg kg^{-1}	milligram per kilo
mg l^{-1}	milligram per litre
mg ml^{-1}	milligram per millilitre
mg O_2 $(Kg\ hr)^{-1}$	milligram of oxygen per kilo per hour
$MgCl_2$	Magnesium Chloride
ml	Millilitre
ml l^{-1}	millilitre per litre
mM	Millimolar
mmol l^{-1}	millimoles per litre
*M*O2	Oxygen consumption
mosm $(kg\ water)^{-1}$	milliosmoles per kilo of water
MT	Metallothionin
mV	milliVolts
Na^+	Sodium ion
NaCl	Sodium Chloride

NaOH	Sodium Hydroxide
ng l^{-1}	nanogram per litre
Ni	Nichel
nm	Nanometre
ORN	olfactory receptor neurones
PAH	polycyclic aromatic hydrocarbons
Pb	Lead
PBDE	polybrominated diphenyl ethers
PBS	phosphate buffer solution
PCBs	polychlorinated biphenyls
PCR	polymerase chain reaction
Pi	Phosphate
PIL	Home Office Personal Licence
PPL	Home Office Project Licence
ppm	parts per million
qPCR	quantitative polymerase chain reaction
RBD	River Basin District
RMR	routine metabolic rate
RNA	Ribonucleic Acid
rpm	revolutions per minute
s	Seconds
S.E.M.	standard error of the mean
SD	standard deviation
SMR	standard metabolic rate
SSRI	Selective serotonin reuptake inhibitor
SVC	spring viraemia of carp
SW	Saltwater
T	Thymine
T_3	Triiodothyronine
T_4	Thyroxine
TBP	Tributyl Phosphate
TE	Tris-EDTA buffer solution
units ml^{-1}	units per millilitre

V	Volts
VHS	viral haemorrhagic septicaemia
Zn	Zinc
Zn^{2+}	Zinc ion
μm	Micrometre
μmol Pi $(mg\ hr)^{-1}$	micromol of Phosphate per milligram of protein per hour

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Chapter 1. General introduction

1.1. Eel biology and life cycle

The European eel, *Anguilla anguilla* (Linneaus, 1758) belongs to the Genus Anguilla, Family of Anguillidae, Order of Anguilliformes and the Class of Actinopterygii (Figure 1.1). It is widespread throughout Europe, ranging from Norway to North Africa (Schmidt, 1909; Tesch, 2003).

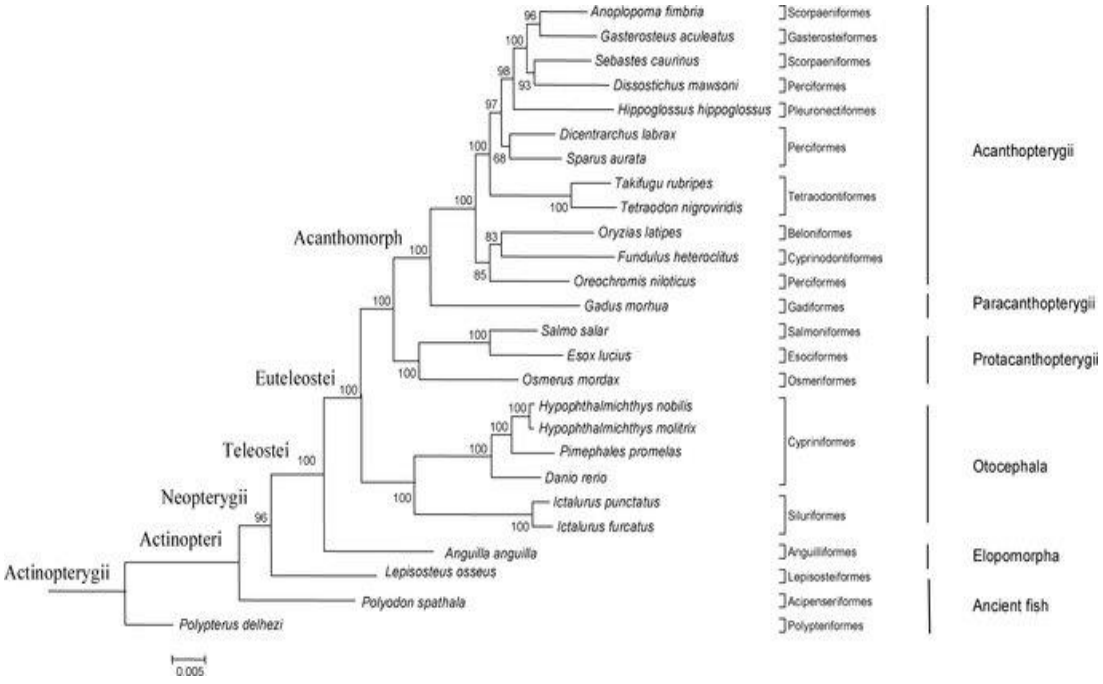


Figure 1.1 European eel classification. (From Zou et al 2012).

It is a diadromous fish that moves between the sea and freshwater at different life stages. Specifically, eels are catadromous spending most of their life in freshwater but reproduce in the sea. The larvae migrate into freshwater, where over a period of several years they reach full adult size before migrating back to sea to spawn. Throughout their life, eels are found in a wide range of habitats from small streams and lakes in freshwater to estuaries and the coastal zone. Their activity is predominantly nocturnal and during the day they prefer to stay covered in crevices or burrowed in sediments when not active (Tesch, 2003). They are carnivorous and the type of food consumed

depends on their life stage, size and availability of their prey. However, they are predatory and predominantly consume live food. The eel life cycle (Figure 1.2) has still not been fully explained.

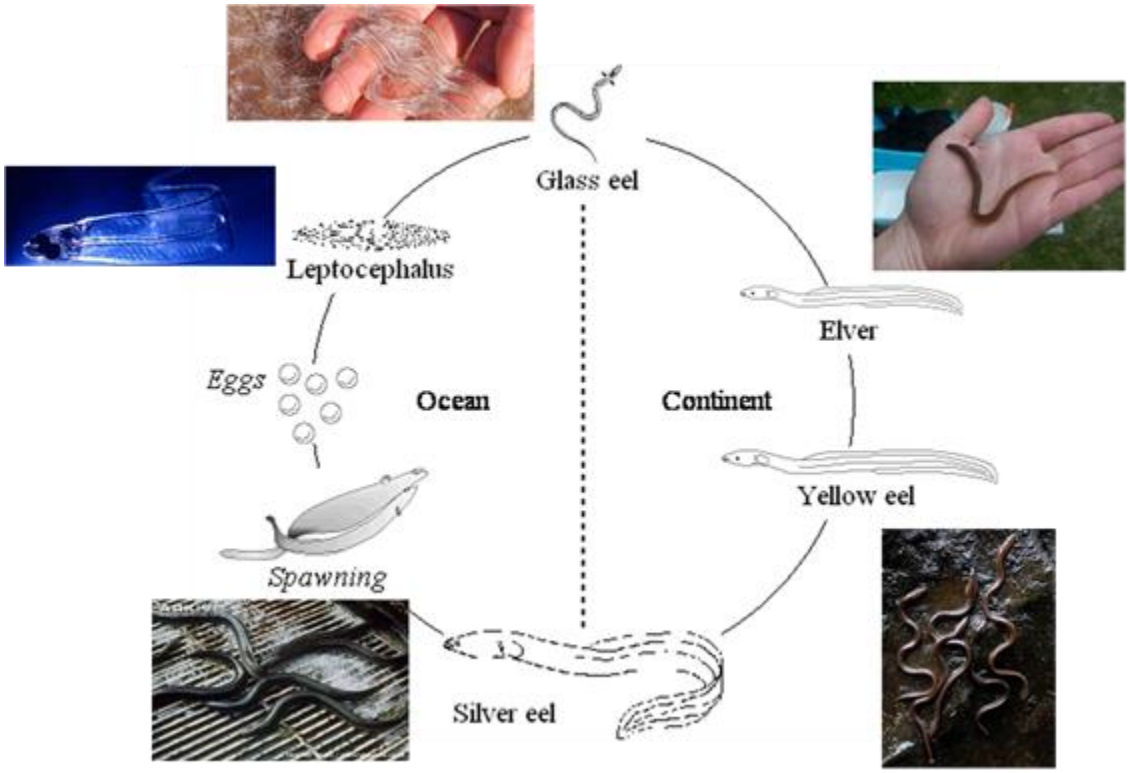


Figure 1.2 European eel life cycle. (Adapted from Dekker, 2002).

Current evidence supports the idea that European eel larvae originate from one spawning stock (panmixia), randomly mating in the Atlantic Ocean (Tesch, 2003; Dekker, 2004; Dannewitz et al., 2005; Als et al., 2011) This is based on the absence of genetic structure between eels from different areas. However, another study, investigating highly polymorphic genetic markers suggests that there is genetic differentiation between populations from the north Atlantic, the Baltic sea and the Mediterranean (Wirth & Bernatchez 2001) characterized by an isolation-by-distance pattern. The genetic structure found for different geographical zones was however considerably smaller than the genetic variation found among temporal samples (Dannewitz et al., 2005) and therefore Dannewitz and colleagues concluded that the hypothesis of an isolation-by-distance is not supported. The eel spawning location is

not known but it is possibly in the Sargasso Sea as determined by the distribution of the smallest eel larvae found (Schmidt, 1923). After hatching the eel larvae (leptocephali) have a leaf shape and a neutrally buoyant gelatinous structure (Miller, 2009) which allows them to drift with currents, but also actively swim with the Gulf Stream towards the European continental shelf where at a size of approximately 70 mm, they metamorphose into glass eels. How long leptocephali take to migrate from the spawning ground to the European continental shelf is still unknown, with some authors suggesting that it could take as little as one year (Bonhommeau et al., 2009) or as long as 2-3 years (Kettle & Haines, 2006). Once glass eels reach the continental shelf, they move towards the coast using "selective tidal transport", where eels are located high in the water column and drift towards the coast during flood tides and rest near the bottom during ebb tides (McCleave & Wippelhauser, 1987). Once they reach the estuaries they start developing skin pigmentation and when the water temperature increases over 10-12ºC (Gascuel, 1986) they may actively migrate against the river flow towards suitable freshwater habitats or remain in the coastal zone for their growing stage. As their estuary entry is dependent on water temperature, the timing of their entry varies with latitude and ranges from winter in the more southern distribution areas to early spring in the northern regions. Along the UK coast, glass eels are first encountered in February, while along the French and Spanish coasts glass eels are found as early as September (Tesch, 2003).

During their growing phase eels are referred to elvers first and yellow eels afterward. The elver stage is reached at an approximate size of 7 cm once the glass eel become fully pigmented (Tesch, 2003). Once they reach an approximate size of 30 cm elvers are referred to as yellow eels. These two phases last between 3 to 20 years depending on gender (5.9 years on average for males and 8.7 years for female) and environmental factors (Vøllestad, 1992), with the life cycle being shorter for populations inhabiting the southern part of their distribution range. After their growing stage, at a size of 35-46 cm for males and 50 to 61 cm for female, adult eels undergo a second metamorphosis from "yellow" to "silver" eels. This metamorphosis, often termed *silvering*, involves morphological and physiological changes that prepare the fish for their trans-oceanic migration to the spawning grounds (Durif et al., 2005). Female eels metamorphose at a greater body length and at an older age than males and both male and female eels initiate silvering at a greater average length as their distance from the

Sargasso Sea increases. Probably it is not the eel size that determines the onset of silvering but rather the eel fat content because of the energy requirement for the long migration (Tesch, 2003). In Euroepan waters, the spawning migration normally begins between September and November depending on the distance between the starting location and the Sargasso Sea (Tesch, 2003). Eels migrate predominantely at night, with peaks of activity between sunset and moonrise, or during dark nights and turbid waters (Tesch, 2003). The morphological changes associated with silvering include a change in the body colour from brown to a clear white belly separated at the lateral line from a dark dorsal region - a counter-shading colouration typical of oceanic pelagic fish (Righton et al., 2012), increase in eye size and a change in the proportions of different eye pigments, regression of the alimentary tract and increase in body fat to a maximum of 25-30% of their body mass (Durif et al., 2005; van Ginneken et al., 2007a; Righton et al., 2012). Physiological changes are linked to the transition from fresh to saltwater, the preparation for the long spawning migration and the onset of sexual maturation (Durif et al., 2005). These changes include increase of gonad weight, increased plasma vitellogenin, haemoglobin, cortisol, testosterone and estradiol. During silvering, thyroid hormone concentrations change with a peak at the beginning of the silvering process and a decrease by the time the fish start their migration (van Ginneken et al., 2007b). The swim bladder develops further vascularization which allows better gas secretion and retention to quickly adapt to changes in buoyancy during the oceanic migration (Righton et al., 2012).

Silver eels leave freshwater systems and swim southward using the Canary and North-equatorial currents and are thought to take 6-7 months to reach the spawning grounds in the Sargasso (van Ginneken & Maes, 2005). Recent tracking studies have indicated that silver eel migrating out of European coasts towards the Sargasso Sea had a horizontal net migration speed of 13.8 km day^{-1} (Aarestrup et al., 2009), which is much lower than the required 35 km day^{-1} necessary to reach the Sargasso Sea for spawning in April. The observed lower travelling speed might be due to a drag effect of the tags used for tracking the fish, or alternatively the eels might gain speed and increase travel efficiency once they enter the south and west flowing currents off the coast of West Africa (Aaresrtup et al., 2009). In addition, eels undertook diel vertical migrations between depths of 200 and 1000m with a distinct night phase in shallow warm water (average depth = 282 ± 138m and temperature = 11.68°C ± 0.48) followed by a steep

dive into cooler areas at dawn maintained during the day at an average of 564 ± 125m and a temperature of 10.12°C ± 0.89° and a later steep ascent at dusk towards shallower areas (Aarestrup et al., 2009). These diel vertical migrations have been explained as a need for the eels to avoid predators, optimize their swimming efficiency and metabolic rate (in warmer waters) and delay the onset of gonad maturation (in cooler waters) (Aarestrup et al., 2009; Righton et al., 2012). However, the presence of the swim bladder parasite *Anguillicola crassus* in most adult European eel could be a contributing factor controlling diel movements. Mature eels have never been observed in the wild, but it is believed that spawning occurs only once and adult eels are supposed to die after spawning (Dekker, 2002).

1.2. Population decline

In the last 40 years all temperate species of eels belonging to the genus *Anguilla* have declined significantly (Casselman & Cairns, 2003). The recruitment of the European eel, *A. anguilla* has declined between 90 and 99% since 1980 (Figure 1.3). In North America, recruitment of the American eel, *A. rostrata* in the Saint Lawrence River has almost ceased although other areas closer to the spawning grounds have shown similar, weaker or no change in their recruitment trends. In Japan the decline in recruitment of the Japanese eel, *A. japonica* has been observed since 1970 and corresponds to about an 80% decline (Dekker & Casselman, 2014)

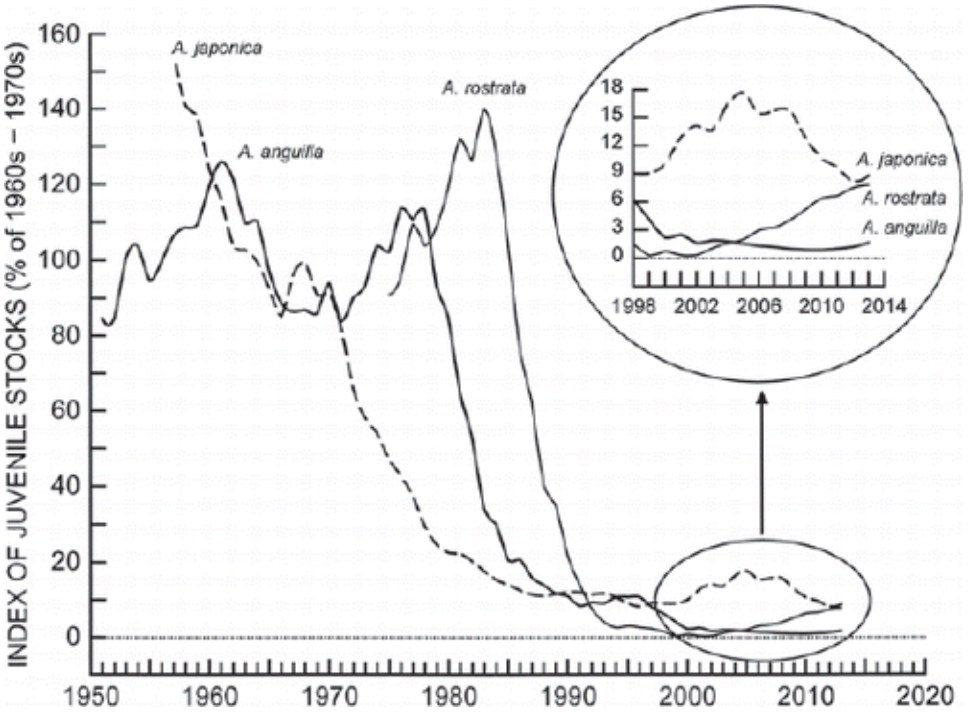


Figure 1.3 From Dekker & Casselman 2014. Time trends in abundance of major juvenile eel stocks of the world. From the 2003 Quebec Declaration of Concern (Dekker et al., 2003), updated: European eel (*Anguilla anguilla*), glass eels, geometric means of available local series from the International Council for the Exploration of the Sea–Working Group on Eels, provided by C. Briand; American Eel (*A. rostrata*), small yellow eels ascending upper St. Lawrence River, from Lake Ontario Management Unit, Ontario Ministry of Natural Resources, provided by J. Casselman; Japanese eel (*A. japonica*), glass eel catches in Japan, from Statistics Bureau, Ministry of Internal Affairs and Communications (prior to 1977 may include young eels larger than glass eels), after 2003 obtained from Fisheries Agency, Japan, provided by K. Tsukamoto. Illustrated using 5-year running means with end-point contractions. Figure prepared by L. Marcogliese.

In Europe, glass eel recruitment has decreased dramatically since approximately 1980 (Figure 1.4), and has reached levels of about 5% of what it used to be before 1980 (Dekker, 2002; ICES, 2013).

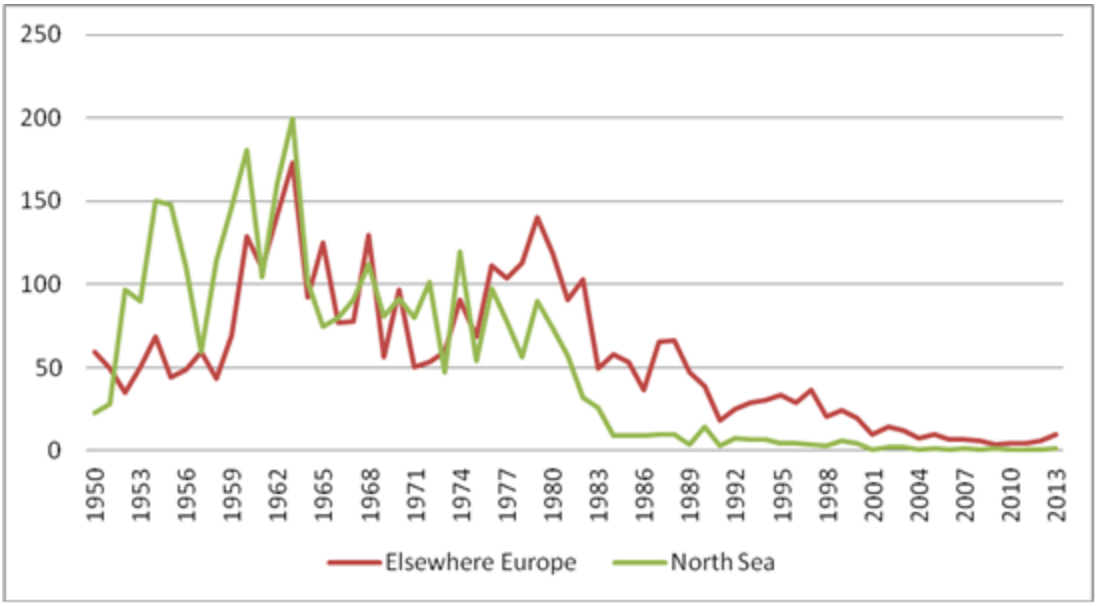


Figure 1.4 European eel recruitment (percentage of exploited glass eels) over the last 63 years. Values presented are the geometric mean of all the time series scaled to their 1979-1994 average (redrawn from ICES advice 2013).

Because of this marked and steep decline, the European eel stock is now considered to be outside safe biological limits (ICES, 2006) and in 2007 the EU implemented an "Eel Recovery Plan" regulation, (EU, 2007) to try to assist in the recovery of the species back to previous sustainable levels. To satisfy the EU Eel Recovery Plan, each Member State is required to develop an Eel Management Plan (EMP UK, 2010) with the aim of achieving an escapement of silver eel to the oceanic spawning population that is no less than 40% of the potential biomass that would be produced in conditions not impacted by anthropogenic disturbance (e.g. fishing, barriers to migration, water quality). As stated in the EMP UK (2010), each member state is required to:

- Set management targets based on assessment of potential silver eel production under conditions of no anthropogenic mortality and high levels of recruitment (pre-1980).
- Estimate present day silver eel production against this target.
- Develop and take management actions necessary to achieve and maintain compliance (40% escapement).

- Collect sufficient data to support the points above and to demonstrate whether compliance will be achieved.

In England and Wales EMPs are set at River Basin District (RBD) level and their aim is to describe the nature of the eel population and fisheries in the RBD, to assess whether the stock meets the 40% escapement target and to present management actions that will ensure compliance and the long term viability of eel populations.

The major factors regulating eel populations are still unknown and until there is an understanding of the factors causing the low recruitment of eels, the success of any management plans and conservation measures may be limited.

The reasons for the eel decline are poorly understood and so far several suggestions have been made. As reviewed by Feunteuen (2002), factors that may have contributed to the eel's decline can be divided into marine or continental (freshwater) factors.

Marine factors refer to global changes that are thought to have provoked a shift northward of the Gulf Stream, making the transoceanic migration of glass eels longer or even impossible (Castonguay et al., 1994; Knights, 2003). A reduction in the ocean productivity has also been suggested as a factor in the reduction in size of glass eel recruited to inland waters (Dekker, 1998; Knights, 2003).

Continental factors can be further divided into:

Barriers to migration. Eels are a diadromous species and therefore need to move upstream during their juvenile stage and later on have to return to the sea for their spawning migration. Obstructions can prevent all or part of a population access to particular areas of a river system and so connectivity is an important aspect in the survival of eels. Many European rivers are highly regulated by dams and therefore an estimate has suggested that of the 123800 km^2 of eel habitat available in Europe at least 33% are not accessible for natural or artificial reasons (Moriarty & Dekker, 1997). This has been particularly studied for glass eels (Briand et al., 2003; Lafaille et al., 2007; Piper et al., 2012). In addition, downstream passage of silver eels through turbines is known to cause high mortalities and can also disrupt downstream migration (Calles et al., 2010).

Fisheries. Throughout Europe, all stages of the life cycle in freshwater have been exploited by commercial fisheries with a total yield in the region of 22000-30000 tons per year (Feunteun 2002). Of these, glass eel fisheries account for 800-900 tons per year which is only 2.7% of the total yield but correspond to more than 2.4 billions of individual eels (Moriarty & Dekker, 1997). The total yield for silver eel fisheries has been calculated at about 2000 tons per year which represent 6.7% of the total population (Moriarty & Dekker, 1997). Yellow eels are exploited for use in aquaculture and restocking programmes. In the UK, reported catches of glass eels have been below 1-2 tons since 2001, compared to 10-70 tons in 1970s and 1980s, and for silver eels the catches since 2001 have been around 29 tons compared to the peak catches of 280 tons in the late 1980s and early 1990s (Aprahamian & Walker, 2008). ICES (2006) now advise that the current fisheries are not sustainable.

Habitat loss has been considerable throughout Europe in the last century due to floodplain dredging and draining and coastal reclamation, destroying suitable habitats for eels. The real extent of suitable habitats that have been lost is not known, but several studies speculate that up to 50-90% of wetlands have been destroyed in the last century in Europe (Feunteun, 2002).

Disease and parasites have been introduced to wild populations, a particular case is the accidental introduction of the swim bladder nematode *Anguillicola crassus* originating from Asia and introduced in Europe in the early 1980s. In as little as a decade this parasite has spread to all water bodies and can be found in 90% of wild eels (Tesch, 2003). This parasite is normally present in low numbers in Japanese eels where it does not cause serious damage, while in European eel the parasite is far more pathogenic (Kirk, 2003) causing thickening of the swim bladder walls and swelling of the swim bladder and abdomen potentially affecting swimming ability and their spawning migration.

Pollutants are widespread in all waters inhabited by eels, and due to their long life and high fat content eels are particularly vulnerable to bioaccumulation of contaminants, especially of the more lipophilic compounds (Robinet & Feunteun, 2002). Direct mortality of eels, due to acute exposure to pollutants, is normally limited to isolated incidents (e.g the Sandoz spill in the River Rhine - Meunier, 1994). Environmental concentrations of pollutants are mostly below acute toxicity levels for eels, however

sub-lethal concentrations can have consequences for the physiology of the eel as it has been demonstrated in other migratory fish species (Madsen et al., 1997; Fairchild et al., 1999; Moore et al., 2003, 2007, 2008; Waring & Moore 2004; McCormick et al., 2005). Further, as silver eels are believed not to feed during their transoceanic spawning migration, the contaminants accumulated in their body fat during the growing stage will be mobilized and become more available when the fat reserves are consumed during their transoceanic migration and gametogenesis (Robinet & Feunteun, 2002). In turn, this may influence the success of the silver eel migration and reproductive success, together with the subsequent larval quality and survival (van Ginneken et al., 2009).

In a review Robinet & Feunteun (2002) argued that freshwater pollution may have a significant detrimental effect on eel health and potentially on reproductive success and survival. Their hypothesis was made in view of the role of several pollutants in interfering with the lipid storage mechanisms which are essential for eels to gain sufficient energy reserves necessary to complete their transoceanic spawning migration and subsequent gametogenesis. In particular, pollutants such as lindane, malathion, endosulfan, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) have been shown to have an effect on thyroid hormones which are intimately linked to the regulation of lipogensis. Further, pesticides such as organophosphates and synthetic pyrethroids have been shown to interfere with lipogenesis via their inhibitory action on the enzyme acetylcholinesterase (AchE) which leads to involuntary and continuous muscular activity resulting in fast lipid mobilization. Another toxic mechanism of contaminants is related to the stress experienced by the eel in relation to poor water quality. As a short term response to stress, teleost increses cortisol production which lead to the lysis of muscle and hepatic lipids and in turn an increase of plasma glucose concentration to provide available energy. In case of long term stress, the first initial increase of cortisol production is followed by hyperactivity of pituitary cells and the inhibition of the cortisol production after several days of stress exposure as it has been observed after exposure to metals, PCBs and PAHs (Robinet & Feunteun, 2002). In principle the excessive lipolysis caused by stress response should delay silvering and emigration until the necessary lipid reserves are restored. However, Fontaine (1994; in Robinet & Feunteun, 2002) showed that successive high concentrations of plasma cortisol for over 7 days

triggered silvering. In addition, eels are long lived species that only reproduce once and therefore do not have the possibility of eliminate accumulated lipophilic compounds in gametes during each reproductive season.

Research studies on the effect of contaminants on eels have so far focused on the potential toxic impact of lipophilic compounds and heavy metals on eel condition, reproduction and embryonic development (Robinet & Feunteun, 2002; Palstra et al., 2006; Geeraerts et al., 2011; Esteve et al., 2012). However, little is known of the potential effects of pollutants during other sensitive life stages of this species, particularly the physiological processes occurring during silvering. Contaminants that affect or modify the silvering process in the European eel may have subsequent impacts on the migratory behaviour and survival during the transition from freshwater and into the marine environment.

1.3. Contaminants in freshwater

As described by the Water Framework Directive, pollution is a deliberate or accidental contamination from human activity that harms ecosystems, human health, material, property, amenities or other legitimate uses of the environment (Environment Agency, 2007). Increasing industrialisation and anthropogenic activity produce many xenobiotics that threaten the environment (Wiegand et al., 2001). The major classes of toxic chemicals that enter the environment are metals, chlorine, cyanides, ammonia, pesticides, polychlorinated biphenyls, herbicides, petroleum hydrocarbons (Heath, 1995) and more recently pharmaceuticals and care products (Lapworth et al., 2012). These compounds have the potential to affect non-target organisms such as fish and can cause a wide range of effects, from disruption in developmental processes to altered reproductive capacity and abnormal behaviours (Trudeau & Tyler, 2007), ultimately resulting in decreasing recruitment to the adult population (Heath, 1995). The contamination of the freshwater environment can generally be defined as either originating from point source pollution or non-point source pollution. Point-source pollution generally originates from wastewater discharged from industrial facilities and municipal sewage (Uriarte & Borja, 2009). This is normally controlled by regulatory processes but is also the main route for household chemicals, including

pharmaceuticals, into the aquatic environment. Legislation directed at pollution control has been in place since the 1970s and includes the Environment Protection Act 1990 (UK), which has helped to decrease the amount of point source pollution from industrial and household emissions. Non-point pollution, often referred to as diffuse pollution, is typically of agricultural origin (e.g. herbicides, pesticides, organic and inorganic fertilisers). Nonpoint source pollution is much harder to regulate and therefore a greater threat to water quality (Faulkner et al., 2000). Although many of the chemicals used in agriculture are applied at specific times of the year, they can enter the aquatic environment at any time as a result of the soil type and distribution and abundance of rainfall. Pollutants derived from land use activities are often persistent and do not degrade readily in surface waters. As a result, they remain toxic for long periods and cause damage to aquatic organisms (Edwards, 2013). The potential threat to fish populations is therefore significant. In England and Wales, as much as 87% of rivers are at risk from diffuse pollution (Environment Agency, 2007) and less soluble components can be washed overland into rivers, especially during heavy rainfall. This often leads to large concentrations of pesticides and chemicals being washed into surrounding waters (Environment Agency, 2007). In the UK, it is reported that 28,000 tonnes of pesticides are used each year and the majority of these chemicals end up in rivers and lakes (Huskes & Levsen, 1997). Once released to the environment, pesticides are often degraded by both biotic and abiotic processes (Stuart et al., 2012). However, the metabolites originating from the degradation can be found in ground waters at higher concentrations than the parent compound (Kolpin et al., 2004) and in some cases are more toxic than the parent compound (Sinclair & Boxall, 2003).

Whether a specific contaminant will have an impact on the eel will depend upon its specific toxicity mode and its temporal and spatial distribution within the aquatic environment. For instance, a specific pesticide that only inhibits ATPase activity within the gills and so reduces the ability of diadromous fish to adapt to saltwater will only have a deleterious effect on the silver eel during its seaward migration, normally in autumn and within the lower reaches of the river system. It's unlikely that the same pesticide will have a negative effect on the feeding behaviour of yellow eels within an upland lake. In the same way, a pesticide or contaminant that only occurs within the coastal and estuarine environments during the spring and which has a toxic effect on swimming ability of fish, will be likely to have a significant effect only on the arriving

glass eels but not on the emigrating silver eel. Therefore, the choice of contaminants in each of the studies carried out in this thesis have been based on the criteria that the toxic mechanism is relevant to a specific life history stage and that they occur at the relevant time and in the relevant location within the aquatic environment. The individual type of contaminants used in the present thesis are described in the individual chapters but a brief general overview of the various classes of contaminants investigated within this work are described below. Figure 1.5 presents an example of water quality monitoring carried out by the Environment Agency (2007).

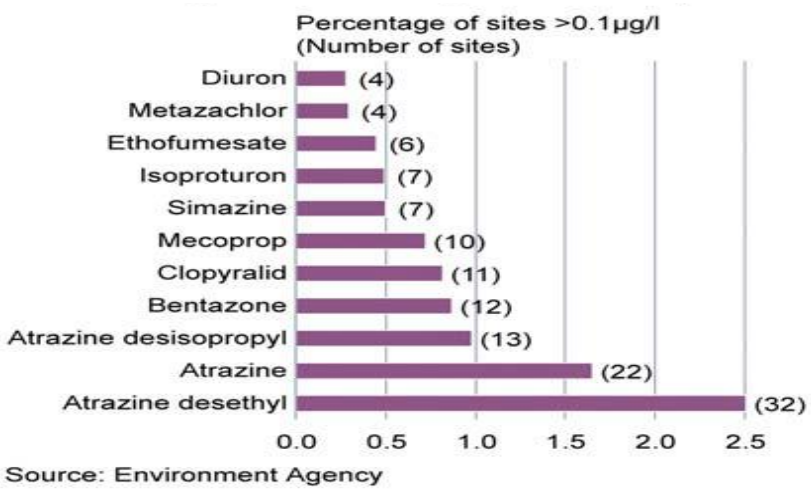


Figure 1.5 Percentage of groundwater monitoring sites which have detected pesticides (Environment Agency, 2007).

1.3.1. Pesticides

A pesticide is any substance or mixture of substances intended for preventing, destroying, or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals, or substances that may be administered to animals for the control of insects, arachnids, or other pests in or on their bodies (FAO, 2002). A pesticide product consists of one or more natural or synthetic active substances co-formulated with other materials. Pesticides can be specifically classified by their use pattern and type of pest they control (Ecobichon, 2001). The target of pesticides can be unwanted plants (herbicides), fungal diseases (fungicide), insects (insecticide), slugs and snails (molluscicide) but (especially from a regulatory

perspective) also attractants, defoliants, desiccants, plant growth regulators and repellents are considered pesticides (Ecobichon, 2001). Currently there are more than 8500 commercial formulations in use of which about 900 are active substances listed in the Pesticide Manual (Jeannot et al., 2000). In the UK, there are currently around 350 ingredients approved for use in agricultural pesticide products (BCPC & CABI, 2010). In an attempt to regulate the presence of pesticides in waters the EU has set a maximum concentration of 0.1 μg l^{-1} for individual pesticides and 0.5 μg l^{-1} for total pesticide present in a sample of drinking water (Jeannot et al., 2000). Data showing the amount of pesticides used for agricultural purposes in Hampshire is presented in Appendix 1 and an extract showing some of the pesticides used in the experimental work presented within this thesis is presented in Table 1.1.

Table 1.1 Estimated annual use of pesticides in Hampshire - kg active substance applied per month (estimate for years 2004/2005). Full table in appendix 1 (Data provided by Environment Agency).

Active substance	chlortoluron	Chlopyrifos	Flusilazole	metaldehyde	pendimethalin
Jan	-	-	-	4.77	55.67
Feb	-	-	8.31	-	1513.31
Mar	-	60.8	459.41	-	1325.99
Apr	-	5.67	101.1	3.59	1466.8
May	-	108.34	820.14	3.66	583.04
Jun	-	4283.55	44.79	115.45	-
Jul	-	350.39	-	-	-
Aug	-	127.99	-	911.14	1.91
Sep	-	-	-	1277.81	2564.58
Oct	4898.54	2.05	34.73	2214.85	6777.54
Nov	8948.17	16.07	1136.12	309.43	25129.67
Dec	-	-	211.03	4.15	7804.44
Annual	13846.71	4954.84	2815.63	4844.84	47222.94

1.3.1.1. Herbicides

Herbicides are chemicals used to manipulate or control undesirable vegetation. They are applied before or during planting to maximize crop productivity by minimizing other vegetation. They can be selective and therefore target only a specific group of plants (e.g. broad leaves) often used to protect particular crops, or they can be generic and target any plant, as it is often the case when used to maintain amenities.

Herbicides are also used in forest management to prepare logged areas for replanting. In this case the total applied volume and area covered is greater but the frequency of application is much less compared to the use for farming (Shepard et al., 2004). Additionally, herbicides are applied to water bodies to control aquatic weeds that would otherwise interfere with water abstraction for irrigation or industrial purposes or with water recreational use (Folmar et al., 1979).

The toxic mode of action of each herbicide determines their effects on the target plants but also the potential effect on non-target organisms. In addition, the method of application would influence the availability and toxicity beyond the target species. Herbicides can act by inhibiting cell division, photosynthesis, or amino acid production or by mimicking natural auxin hormones, which regulate plant growth, and causing deformities in new growth (Ross & Childs, 1996). Methods of application include spraying onto foliage, applying to soils, and applying directly to aquatic systems.

Herbicides can have negative effects on water bodies if they occur in water or sediment at sufficient concentrations. Most commonly, they enter surface water in runoff or leachate, but, because they mostly have relatively low toxicity to fish and invertebrates (EPA, 2010), acute toxicity is likely only when they are deliberately or accidentally applied directly to water bodies. Additional negative effects can occur when herbicides are applied together with other pesticides (Streibig et al., 1998), resulting in additive or synergistic effects. Tests using the earthworm *Eisenia fetida,* show that the herbicide atrazine reacts synergistically with chlorpyrifos, an organophosphate insecticide and the mixture is seven times more toxic than the two individual pesticides (Lydy & Linck, 2003). Additives present in the commercial preparations together with the active ingredient could also be toxic to non-target species, but unfortunately they

are often not specified in the product nor are they considered when the active ingredients are tested (Folmar et al., 1979).

1.3.1.2. Insecticides

Insecticides are substances of chemical or biological origin that are used to control insects by killing them or otherwise preventing them from engaging in behaviours that have deleterious effects on the substrate of interest. They are classified based on their structure and mode of action. Many insecticides act upon the nervous system of the insect (e.g., Cholinesterase (ChE) inhibition) while others act as growth regulators or endotoxins. Insecticides are commonly used in agricultural, public health, and industrial applications, as well as household and commercial uses. Insecticides are applied in various formulations and delivery systems (e.g., sprays, baits, slow-release diffusion) that influence their transport and chemical transformation (EPA, 2010). Mobilization of insecticides can occur via runoff (either dissolved or sorbed to soil particles), atmospheric deposition (primarily spray drift), or sub-surface flow (Goring & Hamaker, 1972; Moore & Ramamoorthy, 1984). Soil erosion from high intensity agriculture facilitates the transport of insecticides into waterbodies (Kreuger et al., 1999). Some insecticides are accumulated by aquatic organisms and transferred to their predators after feeding. Insecticides are designed to be lethal to insects, so they pose a particular risk to aquatic insects, but they also have the potential to affect other aquatic invertebrates and fish.

1.3.1.3. Fungicides

Fungicides are pesticides that kill or prevent the growth of fungi and their spores. They can be used to prevent foliar diseases on vegetable, fields, fruit and ornamental crops and they can also be used to control mould and mildew in other settings than agriculture. Fungicides work in a variety of ways, but most of them damage fungal cell membranes or interfere with energy production within fungal cells. They are more effective as a protective measure rather than curative and therefore they tend to be applied before infection takes place (Battaglin et al., 2011).

1.3.1.4. Molluscicides

Molluscicides are pesticides used against molluscs, they are not specific for particular species but are usually intended for slugs and snails. The most commonly used active ingredient in molluscicides is metaldehyde, which disrupts the mucous membranes of slugs and snails, causing dehydration and eventually death of the mollusc.

1.3.2. Pharmaceuticals

The presence of pharmaceuticals in the aquatic environment has been recognized as a concern for some time (Kolpin et al., 2002; see reviews of Heberer, 2002 and Stuart et al., 2012). The primary route for active ingredients of pharmaceuticals to surface and eventually ground waters is through human excretion, as pharmaceuticals used are often excreted unchanged or only slightly transformed (Heberer, 2002). Additionally, the disposal of unused products, manufacturing residues and the use of pharmaceuticals in agriculture (Stuart et al., 2012) further add to the transfer of these compounds to the environment. Once pharmaceuticals reach water treatment plant, they are often not completely eliminated during the waste water treatment and are also not degraded in the environment (Ternes, 1998; Watkinson et al., 2009), subsequently leaving water treatment via their effluents and reaching streams and rivers. Unlike pesticides that are found in the environment in concentrations that fluctuate during the year (often with peaks in spring), pharmaceuticals are continuously released in the environment via wastewater effluent (Comoretto & Chiron, 2005). Pharmaceuticals commonly detected in aquatic environments are analgesics and anti-inflammatory drugs, antibiotics, antiepileptic drugs, β-blockers, blood-lipid regulators, psychiatric and contraceptive drugs (Jones et al., 2006). A recent review presented data on measured quantities of various pharmaceuticals in water treatment effluents and in freshwater rivers and canals throughout the world. Some of the data for European water bodies are shown in Table 1.2.

Table 1.2 Concentration of pharmaceuticals (ng l^{-1}) measured in the effluent of water treatment plants and in freshwater. Compiled from Calisto & Esteves 2009; Pal et al., 2010; Phillips et al., 2010 and Weinberger II & Klaper 2014).

	Effluent water treatment	Freshwater
Antibiotics		
trimethoprim	99-1264	0-78.2
sulfamethoxazole	91-794	<0.5-4
Analgesic		
naproxen	450-1840	<0.3-146
ibuprofen	134-7100	14-44
ketoprofen	225-954	<0.5-14
diclofenac	460-3300	21-41
salicylic acid	40-190	<0.3-302
Antiepileptic		
carbamazepine	130-290	9-157
B-blockers		
propanolol	30-44	20
Atenolol	1720	314
Blood lipid regulator		
clofibric acid	27-120	1-14
bezafibrate	233-340	16-636
Hormones		
estrone	12.4-196.7	<0.4-33
17β-estradiol	6.2-42.6	<0.1-3.6
17α-estradiol	6.4-12.6	<2
Psychiatric drugs		
butalbital	310-730	5300
diazepam	40-310	33-880
fluoxetine	99	12-1400

Pharmaceuticals are used with the intent to have a biological or physiological effect. They are thus able to pass through cellular membranes and be absorbed, and they are very stable so as not to be inactivated before they have the desired effect (Calisto & Esteves, 2009). Therefore, they are also likely to have effects on non-target organisms once they enter the aquatic environment.

An interesting group of drugs are the psychiatric pharmaceuticals which target the nervous system and are able to alter behaviour and neuro-endocrine signalling (Calisto & Esteves, 2009). This group of drugs include anxiolytics, sedatives, hypnotics and antidepressants, and they are currently among the most prescribed drugs throughout the world (Calisto & Esteves, 2009).

1.3.3. Industrial products

Industrial discharge is, along with agriculture and household sources, a major source of contaminants. Industries produce a large variety of products and frequently chemicals are used during manufacturing. Flame retardants constitute a common group of industrial products that is discharged and therefore measured in the environment.

Flame retardants are used to provide fire safety properties to many different products including furniture, textile and electronics. Flame retardants are categorized as either additive or reactive. Additive flame-retardant chemicals can be added to a manufactured product without bonding or reacting with the product. They are incorporated and dispersed evenly throughout the product, but are not chemically bound to it. Reactive flame-retardant chemicals may be incorporated into the product during manufacture of the plastic raw materials. In general, flame retardants act in one of two ways; either by preventing ignition or preventing the spread of a fire (EPA, 2005). Most flame retardants enter the aquatic environment directly from industrial processes or more indirectly such as the spreading of sewage sludge or leaching from landfill sites containing discarded treated materials. Contamination is particularly high downstream of production sites and industries handling plastic and textiles (Hale et al., 2002; Watanabe & Sakai, 2003; Covaci, 2006).

1.3.4. Metals

Metals are naturally occurring in waters but their levels can be increased through human activities like mining, industrial processes, nuclear power station, agriculture and domestic sewage (Langston et al., 2010). Excess trace metals are a threat to the survival of freshwater fish as high levels of metals discharged in aquatic system might result in the selective elimination of the most sensitive life stages of fish (Bervoets et al., 2005). Chronic exposure to sublethal level of metals has been shown to disturb ion regulation, reduce swimming speed and reduce growth and condition (Sörensen, 1991; Hollis et al., 1999; Alsop et al., 1999; Bervoets & Blast, 2003). For example, in freshwater fish, losses of sodium (Na^+), calcium (Ca^{2+}) and chloride (Cl^-) through passive diffusion from the gills are offset by actively pumping these ions back across the cell membrane (Wood, 2012). Other ions, such as copper (Cu^{2+}) and silver (Ag^+), can compete with Na^+ uptake channels and Na^+/H^+ exchange mechanisms, eventually affecting performance of Na^+/K^+ATPase. Similarly, divalent ions like zinc (Zn^{2+}) and cadmium (Cd^{2+}) can compete with Ca^{2+} transport channels and affect Ca^{2+}ATPase. Processes such as the production of H^+ and HCO_3^- (from CO_2) by the carbonic anhydrase enzyme for use in these pumps may also be inhibited by such metal ions (Baker et al., 2014). Marine teleost gills are also involved in the uptake of Ca^{2+}, and may therefore be similarly affected by dissolved divalent ions. In contrast however, marine fish must drink salt water to replace water lost through osmosis, and gills are mainly responsible for active depuration of Na^+ and Cl^- accumulated via the intestine following drinking (Marshall, 2002). Ingestion of water by marine teleosts means that uptake of dissolved metals and any dissolved contaminant occurs via the digestive tract rather than the gill. This uptake route may be less important in freshwater fish (Baker et al., 2014). In diadromous fish, the interference of metal ions with branchial pumps could cause problems in the acclimation of these species when they move between fresh and saltwater (Shaw & Handy, 2011).

Concerning metal contamination, in the last few decades there has been a general improvement of water quality with a decrease in the measured concentration of most metals (e.g. see Nowen et al., 2001 for Belgium; Owens, 1984 and Jonas & Millward, 2010 for the UK).

Particularly relevant for the work presented in this thesis are the trends in metal contamination in the Bristol Channel and the River Severn estuary which are discussed in detail in Chapter 3.

1.4. Contaminants and diadromous fish

1.4.1. What we know from other diadromous species

In order for diadromous fish to successfully complete their life cycle they must move between the freshwater and marine environments. In the case of two typical UK diadromous species, the Atlantic salmon, (*Salmo salar* L.) and the sea trout (*Salmo trutta* L.) - the migratory form of brown trout, juvenile fish spend the early part of their life cycle in freshwater before emigrating to the marine environment. Salmon and sea trout are anadromous as they reproduce in freshwater but spend their growing phase in the sea. Both species move to the ocean as juveniles (smolts) and undertake a long migration for feeding and growth. The ocean migration brings large growth and fitness benefits to the fish, particularly the female in relation to increased fecundity (Thorstad et al., 2011). The adult fish subsequently return with high fidelity to their home rivers to spawn.

During the smoltification process, salmon and sea trout undergo a number of physiological, behavioural and morphological changes that pre-adapt the fish for a life in the marine environment (Hoar, 1988; Boeuf, 1994; McCormick et al., 1998). Smoltification is characterised by a change to a silvered-coloured body, a reduction in condition factor and a hypoosmoregulatory capacity, concomitant with an elevation in gill Na^+/K^+ ATPase activity (McCormick & Saunders, 1987; McCormick et al., 1987). There is evidence that the ability to live in saltwater occurs within the freshwater zone prior to entry into saline conditions (Moore et al., 1995), which together with an increase in thyroid hormones (Iwata, 1995; Hutchison & Iwata, 1998) and environmental cues (Riley et al., 2002), may trigger seaward emigration. It is during the seaward migration that the smolts undergo olfactory imprinting to their natal stream. Imprinting later allows the fish as adults to return to the home river to spawn

(Nordeng, 1977; Hasler & Scholz, 1983; Nevitt et al., 1994; Dittman & Quinn, 1996: Dittman et al., 1996).

Recent research on the ecotoxicology of diadromous fish has demonstrated that in terms of water quality and pollution, the freshwater and the marine environments cannot be considered in isolation. The conditions experienced in one environment may have significant effects on a fish when it moves to the other. This is particularly true of the Atlantic salmon. Recent research on the effects of diffuse pollution on this species has demonstrated that environmental levels of contaminants experienced by individual fish in freshwater has a direct effect on their physiology and behaviour during the smoltification process. Exposure of smolts to contaminants whilst in freshwater has been shown to affect hypo-osmoregulatory performance, modify the migration of the fish from the fresh to the marine environment and reduce survival once the fish enters the sea (Fairchild et al., 1999; Madsen et al., 1997; Moore et al., 2003, 2007, 2008; Waring & Moore 2004; McCormick et al., 2005). The overall conclusion of this research was that sub-lethal levels of contaminants in freshwater may have a significant effect on the biology of the fish and may be one of the principal factors regulating salmon survival in the sea.

1.4.2. Potential problems for the European eel

The European eel, like the Atlantic salmon, is a diadromous fish and is therefore required to undergo a physiological and morphological transformation during which it develops from the freshwater yellow eel to the migratory silver eel (Durif et al., 2005) and moves out into the ocean. This transformation is very similar to smoltification in the salmon and also involves a number of physiological and morphological changes that pre-adapt the fish for a life in the marine environment as described in Section 1.1. In a similar way to the effects demonstrated on Atlantic salmon smolts, exposure of the adult eel to contaminants in freshwater may modify the silvering process and subsequently have a deleterious effect on migratory behaviour and in particular the ability of the eel to survive the transition between fresh water and the marine environment. Therefore, a major objective of this thesis was to investigate the potential interference of freshwater contaminants on the physiological

changes required in migrating eels to successfully adapt and survive once they migrate out to sea.

Although never studied in the Atlantic salmon, the conditions experienced in the marine environment and coastal waters by diadromous fish returning to freshwater may also have an effect on the successful entry into rivers and streams. Juvenile European eels enter the freshwater environment to undertake their principal growth stage and must move across a saline barrier. This also requires physiological and morphological changes (Ciccotti et al., 1993; Birrell et al., 2000; Jegstrup & Rosenkilde, 2003). Similarly, another objective of this thesis was to investigate the role that contaminants present in coastal and estuarine waters may have on the ability of juvenile eels to adapt and survive as they migrate into rivers and streams.

1.5. Thesis aims

The overall aim of this thesis was to investigate the impact of specific environmental contaminants on the transitional stages of the European eel, in particular during the transition between life stages occupying different environments (e.g. freshwater and marine). The aim of the work also examined the potential impact of specific contaminants on the feeding behaviour of the freshwater stages of the eel.

1.6. Outline of thesis

Chapter 1 provides an introduction to the biology and life cycle of the European eel and the potential reasons for the recent decline observed in eel populations. It further describes the management decisions taken at European level in order to assist in the recovery of the eel population. It then highlights the possible reasons for the decline and focus on pollution as one of the potential cause. It describes the chemical classes tested in the work presented in the subsequent chapters.

Chapter 2 describe the general methods used for sourcing the eels and maintaining the fish within the laboratory, sampling and tissue analysis. It also describes techniques used in the studies to investigate specific research questions.

Chapter 3 describe experiments carried out on juvenile stages of eel when the animals move from the estuary and into freshwater. Two experiments were carried out for this section. The first looked at the effect of tributyl phosphate exposure on glass eel survival and adaptation to freshwater. The second looked at the effect of individual metals or a mixture of metals on glass eel survival, adaptation to fresh water and DNA integrity.

Chapter 4 examines the effect of contaminants on the growing stage of eel. The first experiment investigates the effect of the sediment bound flame retardant hexabromocyclododecane (HBCD) on elver survival and growth. The second experiment looks at the effect of sediment bound and/or food derived metals on elver survival, growth, DNA integrity and gene expression. The third experiment investigated to potential effect of fenitrothion exposure on yellow eel olfaction, while the last experiment looks at the effect of atrazine on eel olfaction.

Chapter 5 addresses the interaction between freshwater contaminants and silver eel physiology and migratory behaviour. The first experiment looks at the survival and saltwater adaptation of silver eel exposed to fenitrothion in fresh water. The second experiment investigates the effect of Tributyl phosphate (TBP, a flame retardant and plasticizer) exposure on survival, saltwater adaptation and downstream migration of silver eel. The third experiment examines the effect of a pesticide mixture on silver eel survival, adaptation to saltwater, metabolic cost and downstream migration. The last experiment investigates the metabolic cost of exposure to the antidepressant fluoxetine of saltwater adapted silver eel.

Chapter 6 discusses the overall results obtained during experiment and their relevance to the decline of the European eel.

A summary of all the contaminats investigated, the eel life stage investigated and the exposure route used and the time and location of the study is presented in table 1.3.

Table 1.3 Summary of experiments performed for this thesis.

date	location	life stage	contaminant	exposure route
Nov-09	Cefas laboratory	migrating	fenitrothion	water
Jan-10	Cefas laboratory	adult	fenitrothion	water
May-10	Cefas laboratory	glass eel	TBP	water
Nov-10	Danish laboratory	migrating	TBP	water
Nov-10	Randers Fjord (DK)	migrating	TBP	water
Jun-11	Cefas laboratory	elvers	HBCD	sediment
Jul-11	Cefas laboratory	adult	Atrazine	water
Nov-12	Cefas laboratory	migrating	pesticide mixture	water
Nov-12	River Avon (UK)	migrating	pesticide mixture	water
Jan-13	Cefas laboratory	migrating	fluoxetine	water
Jun-13	Cefas laboratory	glass eel	metals (Cu, Pb, Zn, Cr)	water
Nov-13	Cefas laboratory	elvers	metals (Cu, Pb, Zn, Cr)	sediment - food

Chapter 2. General methods

2.1. Chemicals and fish source

All chemicals, unless specified otherwise were purchased from Sigma-Aldrich, UK.

Unless otherwise stated, glass eels and elvers where purchased from Glass Eels UK Ltd (Gloucester). The glass eels were caught in the estuary of the River Severn during the spring run (February - April), and the elvers were part of the Company's growing-on programme. Glass eels and elvers were supplied in water filled boxes and transported to the Cefas Lowestoft Laboratory. Once in the laboratory they were immediately transferred to 100 litre tanks with flow through seawater (for glass eels) or freshwater (for elvers) with a constant flow of 1 l min^{-1} and an air supply to provide gentle aeration. Seawater was pumped from wells positioned at a depth of 2 m from the low water mark on the beach adjacent to the Cefas Lowestoft Laboratory (coordinates 52.472°N, 1.740205°E). Freshwater was obtained by de-chlorinating tap water. Samples of all water supplies (tap water, dechlorinated tap water and seawater) to Cefas Lowestoft Laboratory were sent to National Laboratory Services for chemistry analysis and the results are presented in Appendix 2.

Unless otherwise stated, adult eels were purchased from a commercial fisherman operating in both the River Avon (Hampshire) and the River Stour (Dorset). The eels from the River Avon were caught by fyke nets while the fish from the River Stour were caught using an eel rack at Longham (coordinates 50.7863°N, 1.90673°W). Fish were caught while emigrating downstream during October and November. Fish collected over consecutive nights were held in tanks in the river until collection (3 - 10 days following capture). Fish were placed in water-filled polythene bags in groups of 5 - 10 individuals and oxygenated using battery operated aquarium aerators. Each bag was placed in a dark plastic container to reduce stress levels. Fish were then transported to the Cefas Lowestoft Laboratory and on arrival immediately distributed into tanks (550 l) with a constant flow of de-chlorinated tap water, an air supply and one or two hiding features (Figure 2.1).

Figure 2.1 Eel holding tank.

Both juvenile and adult fish were kept in a naturally simulated photoperiod (longitude 52°N) and were fed to satiation daily with commercial food pellets of appropriate diameter for their size.

2.2. Fish sampling

All experiments described were carried out under a Home Office Animals (Scientific Procedures) Act 1986 Personal Licence (PIL 80/10073). All the work was carried out under an appropriate Project Licence held by Dr Andy Moore (PPL 80/2174 and 70/7588). At all times the eels used in the experiments were maintained under carefully controlled conditions that ensured their welfare. Prior to sampling, eels were anaesthetised with 2-phenoxyethanol (2-4 ml l^{-1}) dissolved in water. The eels were able to detect the anaesthetic in the bath and would often lift their heads out of the water. Therefore, all procedures with adult eels were undertaken in a suitable sized plastic bag which could be closed at the top without any air left over the water, ensuring that the eel inhaled the anaesthetic. Fish were considered to be fully

anaesthetized when there was a total loss of muscle tone and total loss of equilibrium. This was usually achieved within 3 - 6 minutes.

In juvenile eels, the wet weight was measured to the nearest 0.01g and the length was measured to the nearest 0.1cm. In adults, weight of fish, liver and gonads were measured to the nearest 0.1g. Liver and gonad weights were used to calculate hepatosomatic (HSI) and gonadosomatic (GSI) indexes respectively. HSI was calculated as 100 x [liver weight (g)/fish weight (g)], and similarly GSI was calculated as 100 x [gonad weight (g)/fish weight (g)]. Fish length was measured on a measuring board to the nearest cm and eye horizontal and vertical diameters were measured with a digital calliper to ± 0.1 mm. Individual length and weight were used to calculate the Condition Factor (CF) as 100{body weight (g)/ body length (cm)^3}. Eye diameters were used to calculate the Eye Index (EI) according to Pankhurst (1982).

$$EI = \left[\frac{\left(\frac{A+B}{4}\right)^2 \pi}{L}\right] 100$$

A=horizontal eye diameter

B= vertical eye diameter

L=total fish length

Fat content of adult fish was measured by averaging four external readings taken with a Distell fat meter at successive positions along the body of the eel from the head to the tail region. The meter automatically calculated the percentage of fat in relation to the amount of water contained in the measured sample. The instrument calculated the fat percentage using an eel specific calibration relationship (see www.distell.com).

2.3. Tissue analysis

2.3.1. Measurement of gill and kidney Na^+/K^+ ATPase.

Gill samples of juvenile fish were obtained by taking the whole body section containing the gill arches (See Figure 2.2).

Figure 2.2 Glass eel section (between vertical blue bars) utilized for gill ATPase assay.

In adults, the third gill arch was removed from the left side of the head, and a kidney sample was taken from the distal portion of the organ.

Once collected, gill and kidney samples were placed in a micro-tube with 0.75 ml of cold SEI buffer (0.3M sucrose, 20 mM Na_2EDTA, 0.1mM imidazole; pH adjusted to 7.1 with few drops of 1M HCl) and immediately frozen in liquid nitrogen and later transferred to a -80ºC freezer until assayed (Zaugg, 1982). Prior to protein determination, all the samples were prepared using a Biofuge 15R centrifuge set to the following parameters: temperature = 10ºC, time = 8 minutes and speed = 245 *g*. After centrifugation the supernatant was discarded and the bottom pellet re-suspended in 750 µl of SEI buffer with deoxycholic acid (2.41mM). The tissues were then manually homogenized in a conical glass homogeniser with a teflon pestle until all filaments or tissues disintegrated. The resulting homogenate was then centrifuged again at 10ºC for 8 minutes at 480 *g* for gill tissues and at 978 *g* for kidney samples. After centrifugation the pellets at the bottom of the tube were discarded to waste and the supernatant was kept on ice and used for the determination of protein content and ATPase activity.
The samples protein content was estimated with a commercial Pierce BCA™ Protein Assay Kit (Perbio Science, UK), consisting of BCA reagent A, BCA reagent B and albumin standard, 2 mg ml^{-1} following the manufacturer instructions.

The value obtained for the protein content of each sample, was then used to prepare a solution of sample diluted with SEI buffer in order to obtain a solution containing 1 mg ml^{-1} of protein. This standardized solution of each sample was then used to determine the Na^+/K^+ ATPase activity.

Gill and kidney Na^+/K^+ ATPase activity was assayed according to the method described by Schrock et al. (1994) with the enzyme activity shown as μmol Pi (mg protein×h)$^{-1}$. Briefly, 10 μl of the sample solution was added into each well of a 96 well microplate. Then 75 μl of solution AA (1 ml ATP solution – 33.39mM; pH 7.0 adjusted with few drops of 1M NaoH and 6.5 ml of solution A - 155.2mM NaCl, 23.02mM $MgCl_2$, 75.12mM KCl, 115.01mM imidazole; pH adjusted to 7.0 with few drops of 1M HCl) or solution AB (same as for AA but solution A also contain 0.72mM ouabain) is added, and incubated at 37ºC for 10 minutes. The reaction was stopped by adding 200 μl of a 1:1 mixture of 10% trichloric acid and "colour reagent" (9.20mM ammonium molybdate, 0.66 mM sulphuric acid and 0.33mM ion sulphate). After 30 minutes of incubation at room temperature the Pi produced was determined spectrophotometrically at 630 nm using BioRad benchmark microplate reader and calculated against the reading of phosphate standard (PO^{3-}) obtained commercially and used undiluted and the SEI buffer as blank.

2.3.2. Measurement of plasma ions, osmolarity and glucose

Prior to blood sampling, syringes, needles and micro-tubes for blood collection were rinsed with heparin solution (500 units ml^{-1} dissolved in 0.9% saline – NaCl dissolved in ultrapure water). Blood was collected from the caudal vein of anaesthetized adult fish using a heparinised 5 ml syringe with a 25G needle and then transferred in a 2 ml heparinised centrifuge tube. Blood was then centrifuged at 4472 *g* for 3 min. After centrifugation the plasma was transferred with a pipette (Gilson) and a clean tip (Gilson) for every sample in aliquotes of 100 μl in 0.5 ml labelled micro-tubes and stored in a -20°C freezer until analysis.

Osmolarity was measured using 100 μl aliquots by means of an automatic Osmometer (Löser, Berlin, Germany) displaying the mosm as per kg of water. The instrument was

calibrated according to the manufacturer instruction with deionized water and a standard 300 mosm (kg water)$^{-1}$ (Löser, Germany) solution.

Plasma ions: Chloride ions were measured with a Jenway PCLM3 Chloride meter. 20 µl of plasma were used for the measurement of mmol l^{-1} of chloride in the sample. The sample was added to 20 ml of "combined acid buffer" (Reagecon, Ireland) after calibration with 100 mmol l^{-1} of chloride standard (Jenway, England). Sodium and potassium were measured with a Sherwood Flame Photometer after diluting the plasma 26 times for potassium and 773 times for sodium.

Glucose was measured with a colorimetric assay. 50 µl of each sample was diluted 10 times with deionized water, then 3 ml of colour reagent (0.12M PBS with 6.8 units ml^{-1} Peroxidise enzyme**,** 6.5 units ml^{-1} Glucose Oxidase enzyme and 0.3 mg l^{-1} ABTS) was added to the samples and incubated at 37°C for 30 minutes. Absorbance was measured with a spectrophotometer at wavelength of 440 nm. Glucose concentrations were calculated from a standard curve of D-glucose in concentrations ranging from 1.4 mM to 0.09 mM.

2.3.3. Comet assay

The Comet Assay (single-cell gel electrophoresis) is commonly used in ecotoxicological studies for assessing DNA damage as strand breaks (Guilherme et al., 2010) and has been successfully applied to toxicological studies on eels (Nigro et al., 2002). The loss of DNA integrity can have very serious consequences for organisms as they may lead to mutations, chromosomal aberrations, birth defect and long term effects such as cancer (Nigro et al., 2002). DNA strand breaks are an early sign of damage which might still be subject to a repair process (Guilherme et al., 2010) and therefore may not necessarily be permanent.

The Comet assay was performed on red blood cells from glass eels and elvers. For each fish, blood was obtained from deceased animals by severing the tail 10 mm from the tip and collecting a drop of blood on a glass microscope slide. From the slide 2µl of blood was collected and diluted in 498 µl of PBS. 10 µl of the blood and PBS solution was then added to 160 µl of melted LMP Agarose (Trevigen, UK). For each fish, 3 aliquots of 50µl of sample in agarose were placed onto each of the circles of a 20 well comet assay slide (Trevigen, UK). The slide was kept on ice and in the dark

until all wells were loaded with samples. Once all wells were completed the slide was kept covered in the fridge to allow the agarose gel to set. Once set, the slide was transferred in a glass beaker and covered with 100 ml of cold complete lysis solution (90 ml lysis solution from Trevigen, UK, 10ml DMSO and 1ml Triton X) and left in the fridge for 45-60 min. At the end of the lysis stage slides were taken out of the beaker and washed with ultrapure water then dried. At this point the slides were transferred to a comet assay electrophoresis chamber and covered with Electrophoresis solution (300 mM NaOH, 1 mM EDTA) and left at 4-6°C for 30 min. At the end of these 30 min the electrophoresis chamber was set at 30V, 300mA and run for 30 min. Once the electrophoresis was completed, slides were removed, washed carefully with ultrapure water and transferred in absolute ethanol for 15 min. After dehydration in ethanol the slides were tapped dry and stored in a dark box until microscope analysis. Comet slides were analysed using the software "Comet IV". Before observation each well was stained with 15 µl of DNA dye - SYBR gold (Invitrogen) diluted 200 times in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). 50 random and isolated cells were selected and measured in each well. An example of cells and their Comet tails is shown in Figure 2.3. The measures taken included the tail intensity which is the relative intensity of the comet tail to the head and reflects the number of DNA breaks, the tail length and the tail moment. The tail moment combines the amount of DNA in the tail with the distance of migration (i.e. the severity of damage) and is calculated multiplying the percentage of DNA in the tail with the tail length.

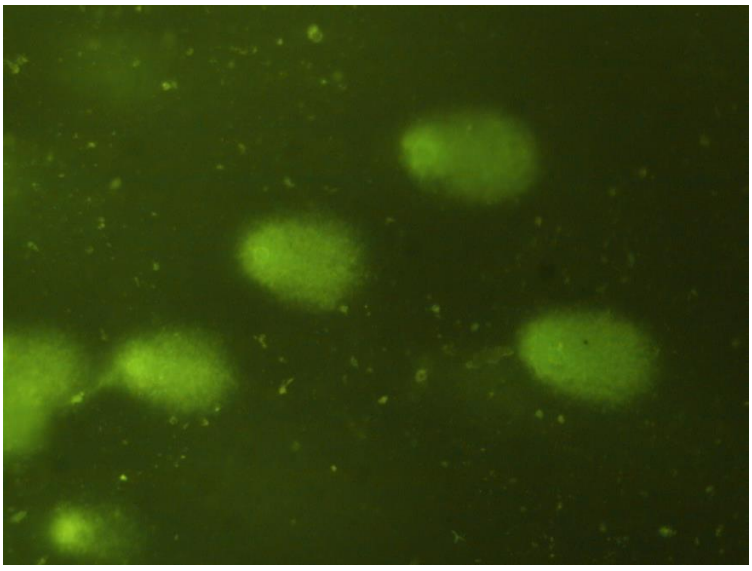


Figure 2.3 Image of stained DNA showing the comet on one side of the nuclei.

2.3.4. Real Time qPCR

Small liver biopsies (10-15 mg) were homogenized in 2 ml tubes with glass beads and solution TR1 with β-mercaptoethanol following UltraClean® Tissue & Cells RNA Isolation Kit (Mobio) instructions. Homogenization was achieved using a vortex mixer with 2 x 60 s cycles. RNA was extracted from homogenised tissue following the kit instructions and including the optional genomic DNA removal, achieved by on-column DNase I Kit (Mobio) as recommended by the manufacturer. Extracted RNA was quantified using a nanodrop (Thermo Fisher Scientific). Reverse transcription was carried out with SuperScript™ III Reverse Transcriptase (Invitrogen) following the manufacturer's guidelines and the cDNA obtained used to prepare a dilution 1:4 of cDNA:water and then used as a template for quantitative PCR. The genes analyzed were metallothionein (MT) and cytochrome P4501A (CYP1A) in addition to two housekeeping genes, elongating factor-1 (EF1α) and Ribosomal protein L13a (L13) to provide internal standard for CYP1A and MT expression (Maes et al., 2013). Primers for the genes investigated are listed in Table 2.1.

Table 2.1 Specific primer pairs for the four target genes tested in European eel. (Modified from Maes et al. 2013).

Gene	Primers	Sequence (5'-3')	Source/Reference
EF1α	EF1-F	GGCTGGTGGTGTAGGTGAGT	EU407825
EF1α	EF1-R	TAAGCGCTGACTTCCTTGGT	
L13	L13-F	AAAGGAAGCGTATGGTGGTG	Coppe et al. 2010
L13	L13-R	CGGTCTTCTTCTTGCCGTAG	
MT	MT-F	TGCACTACGTGTAAGAAAAGCTG	Pierron et al. 2007
MT	MT-R	ACACATACAATAAACCCAACACAAATGA	
CYP1A	CYP1A-F	CGCTCCTTCTCCACCATCA	Aubry et al. 2007
CYP1A	CYP1A-R	CAGGATTGCCACTGCCCGC	

All genes were analysed with a SYBR green PCR master mix (Go Taq® Promega) using an Applied Biosystems StepOne Plus System and software version 2.3 (Applied Biosystems, Foster City, CA, USA). Each 20 µl reaction contained 2 µl dilute cDNA, 1X SYBR master mix (Promega, UK) and primers at 0.3 µM. The reaction conditions were as follows: 2 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C,

with fluorescence measured at the end of each 60 s elongation step. In addition, each amplification was confirmed by melt curve analysis.

PCR efficiencies were calculated with LinRegPCR (Ruijter et al., 2009). Relative expression (relative to a control) was determined by REST software (Pfaffl et al., 2002).

2.4. Dosing protocol

A concentrated stock solution was prepared for each compound tested. These were made up by measuring amounts of chemicals on a weighing boat to the nearest 0.001 g (Mettler Toledo AG64) which was then dissolved in either distilled water, ethanol or acetone using a magnetic stirrer (Stuart SB162) at room temperature.

Dosing in the tanks was achieved either in static or in flow through conditions.

For experiments run in static conditions, the water inflow to the holding tanks was turned off at the beginning of the experiment and a suitable amount of concentrated stock solution was added in the tank in order to achieve the desired testing concentration in the total tank water volume. To maintain good water quality, part or all of the water was replaced with fresh water spiked with fresh testing compound every 2-5 days depending on the number of animal and their biomass and whether feeding was taking place or not during the exposure experiment.

For experiments run in flow through conditions the desired testing concentration was obtained and maintained using a peristaltic pump (Watson Marlow 205U) and tubing of 0.88mm bore (orange-orange) constantly pumping a dosing solution into the tank. The dosing solutions were obtained diluting the stock solution with tank water.

The following calculations were used to determine the concentration of dosing solution required to reach the testing concentration in the fish tank:

$$S = \left(\frac{Outflow}{Inflow}\right) * F$$

Where:

S = concentration of dosing solution (µg l^{-1})

Outflow = flow rate out of the tank (l min^{-1})

Inflow = flow rate into the tank (l min^{-1})

F = final concentration in the tank (testing concentration)

This was derived considering that the final concentration F need to remain constant and therefore the same amount of contaminants that leave the tank must be replaced by a same amount entering

Weight entering = weight leaving

$$Inflow * S = Outflow * F$$

$$S = \left(\frac{Outflow}{Inflow}\right) * F$$

The peristaltic pump (Fig. 2.4) was set at 40 rpm which, related to the size of tubing used, was equivalent to 1.4 ml min^{-1}. The dosing solutions were made up in 3 or 5 l glass beakers using dechlorinated water and were made fresh and replaced every 24 hr (3 l) or 48 hr (5 l). The beakers were labelled and covered to minimize evaporation and the potential for cross-contamination. Mixing of the dosing solution entering the tank and the water in the tank was achieved by aerating the water in close proximity to the tube feeding the dosing solution into the tank.

Figure 2.4 Peristaltic pump set up for dosing experiment in 8 tanks.

2.5. Behavioural observations

Behavioural observations were undertaken in individual glass tank holding juvenile eels by recording the number of swimming fish in a 10 s snapshot. Those observations were carried out every morning (Monday to Friday) between 10:00 and 12:00 for the duration of the experiment. Tanks were observed in random order starting at least 15 min after the observer had entered the experimental room to allow the fish to resume normal behaviour after the potential disturbance induced by the entrance of the observer. Observations were carried out from a distance of about 1.5 m, avoiding standing directly in front of the tank that was observed. Observations were always carried out before any routine husbandry procedure (e.g. feeding, tank cleaning and water replacement) was carried out. For each tank the number of eels that were actively swimming in the tank was recorded. All eels either hiding in the shelter provided or in the sediment (depending on the experiment type), or just lying still on the bottom of the tank were considered as inactive and not accounted for.

2.6. Electro-olfactogram (EOG)

The impact of contaminants on the eel sense of smell was studied using the underwater electro-olfactogram (EOG) recording technique. The EOG is an established technique for measuring peripheral olfactory function in fish and other vertebrates (for review see Scott & Scott-Johnson, 2002). The odour evoked EOG is an extracellular field potential that consists of a large negative voltage transient measured with an electrode near the surface of the sensory olfactory epithelium of the fish. The amplitude of the EOG reflects the summed electrical response of many olfactory receptor neurones (ORN) as they bind the dissolved odorant. The EOG is a robust and direct measure of the ORN function in intact animals. As a result, the technique has been widely used to investigate olfactory signal transduction (Baldwin & Scholz, 2005). The EOG technique is a particularly potent tool to examine the impact of waterborne contaminants on fish olfactory function (Moore, 1994; Moore & Waring, 1995; 1996; Moore & Lower, 2001; Moore et al., 2003: see Tierney et al., 2010 for review) and is now widely used in a number of toxicological research.

Eels were anesthetised with 2-phenoxyethanol (2 ml l^{-1}) and the skin and the cartilage removed to expose the olfactory rosettes (see Figure 2.5).

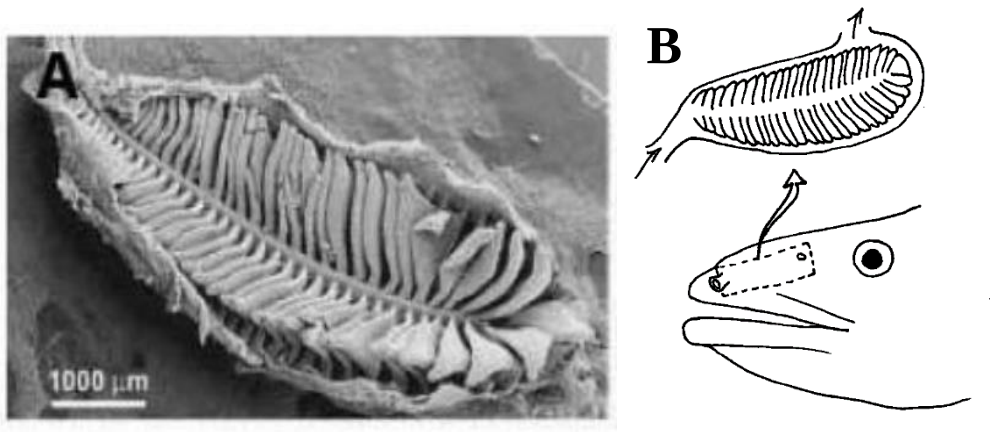


Figure 2.5 A: scanning electron micrograph of the olfactory rosette of the European eel (from Hansen and Zielinski, 2005). B: diagram of position of olfactory rosette in eel heads.

The eels were then immobilised with an intramuscular injection of gallamine triethiodide (0.3 mg kg^{-1} body mass) and placed in a V-shaped clamp in a Perspex flow-through chamber. The gills were constantly perfused with water containing 2-phenoxyethanol (2 ml l^{-1}). Electrophysiological recordings were made by using glass pipettes filled with a saline-agar solution (2%) bridged to an Ag–AgCl electrode (Type EH-3MS, Clark Electromedical Instruments) filled with 3 M KCl. The electrode (tip diameter of 90–100 μm) was produced using a Narishige PC10 electrode puller. The tip of the electrode was placed close to the olfactory epithelium at the base of the largest lamellae in the centre of the rosette. This was where the maximum response to 10^{-3} M L-glutamine, (mean 1.7 ± 0.3 mV) and minimum responses to dechlorinated water (mean 0.05 ± 0.01 mV) were obtained. An Ag-AgCl reference electrode was grounded and placed lightly on the skin of the nares of the fish. The signal was amplified using a Neurolog Systems DC preamplifier (Digitimer Ltd.) and displayed directly and subsequently stored on a Yokogawa DL1520L Digital Storage Oscilliscope for later analysis. Previous studies on salmon using the same electrophysiological technique have indicated that there were no changes in responsiveness to odorants by the parr during the duration of the experiments (5–6 hr) (Moore & Lower, 2001; Moore et al., 2003).

2.7. Respirometer

Metabolic rate was indirectly determined by measuring oxygen consumption – $\dot{M}O_2$ - (Clarke & Johnston, 1999) in a swim tunnel respirometer (See figure 2.6 for a schematic diagram). Oxygen measurements were made using the intermittent flow respirometer described by Wright et al. (2014).

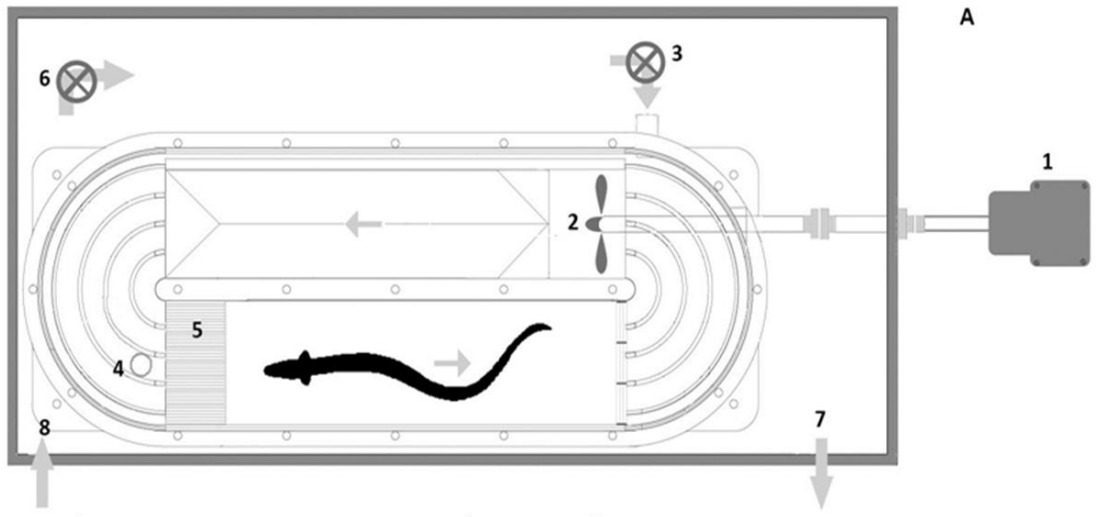


Figure 2.6 Diagram of a swim tunnel respirometer. **Adapted from Methling et al. 2011. 1. Motor; 2. Propeller; 3. Flushpump (inlet); 4. Mixing pump; 5. Honeycomb; 6. Flush outlet; 7. Outlet from tank to drain; 8. Inlet to tank from seawater supply. Arrows indicate water flow.**

Briefly, the respirometer (swim chamber section of 25 × 25 × 87 cm) was submerged in an outer tank, which measured 232 × 95 × 70 cm having a total water capacity of 187 l (Loligo Systems, ApS). The outer tank served as a source of aerated water used for flushing (flush pump, Eheim, 20 l min^{-1}) the respirometer (swim chamber) after each 'closed' measuring phase. Water quality in the outer tank was maintained by providing an inflow (10 l min^{-1}) of fresh ambient seawater. The water in the outer tank was kept fully aerated. Each swim trial was broken down into 'measurement', 'flush' and 'wait' phases. During the measurement phase, the oxygen tension of the water in the swim chamber was recorded using a galvanic oxygen electrode, while the swim chamber was completely closed from the outer tank. MO_2 was calculated from the rate of decrease in oxygen tension. Subsequently, the swim chamber was flushed with aerated seawater from the outer tank to replenish oxygen levels (flush phase), and then a 'wait' phase enabled the oxygen levels to stabilise before the next measurement phase. During swim trials, chamber flushing and the recirculation valve were controlled through an interface (DAQPAC- G1X, Loligo Systems) connected to a PC running AutoResp™ software (Version 1.6, Loligo Systems). Oxygen tension within the swim chamber was measured using a mini dissolved oxygen galvanic cell probe suspended into the water current of the respirometer, which was connected to the DAQ interface, and oxygen saturation data was calculated using AutoResp™. To avoid

effects of temperature or feeding activity, the respirometer was run with ambient seawater, which was the same as used in acclimation tanks and experimental tanks for the fish. Eels were not fed during the five days exposure period nor while in the respirometer. Water speed in the respirometer was low (0.23 m sec^{-1}), the "measurement" phase was 2500 s long, the "flush" phase was 399 s and the "wait" time was of 1 s. Fish were transferred individually to the respirometer, and only one fish was tested each day. Fish were moved in the chamber between 10:00 and 12:00 each morning and measure of oxygen consumption started immediately and continued for 24 hr. When a fish is transferred to a new environment, such as a respirometer, an oxygen debt due to anaerobic exercise during handling and the unfamiliar surroundings results in an initial elevation of MO_2. This may last several hours and during this period a gradual decrease in MO_2 can be observed. Following this period, MO_2 stabilizes with a distinctive lower level. This lower level is the Standard Metabolic Rate (SMR) of the fish, while higher measurements are due to random activity and can be considered Routine Metabolic Rates (RMR) (Schurmann & Steffenson, 1997). SMR for each fish was calculated as the mean of the six lowest measurements of *M*O2 as described by Schurmann & Steffenson (1997) and the RMR was calculated by averaging all the MO_2 recorded after the initial settling period.

Oxygen consumption was calculated from the rate of decline in oxygen tension, the volume of the swim chamber and the solubility of oxygen in seawater at the experimental temperature (Schurmann & Steffensen, 1997; Lee et al., 2003). Values for MO_2 in mg O_2 $(Kg\ hr)^{-1}$ were therefore recorded every 42 min (the duration of the flush cycles). MO_2 values were then converted from milligrams of O_2 per kilogram per hour to micromoles of O_2 per kilogram per hour before further processing.

2.8. Acoustic telemetry

The migratory behaviour of silver eels as they moved from freshwater into the marine environment was investigated using acoustic telemetry. Sound propagates particularly well through open water and acoustic tracking techniques are often applied to free ranging aquatic animals (for a review see Hussey et al., 2015). Acoustic telemetry was chosen over alternative technologies such as radio telemetry as the signals produced by the tags are detected within both fresh and saline water, which is a requirement

when studying the movements of fish through estuaries. Radio signals from tags are rapidly attenuated through sea water and will not normally penetrate more than a few meters of fresh water. Acoustic tags can be located more precisely than radio tags making them more suitable for high resolution tracking. However, acoustic signals do suffer severe attenuation in shallow or fast-flowing water, aquatic vegetation or turbid conditions, where acoustic noise, entrained air bubbles and suspended solids can drastically reduce range.

The use of acoustic transmitters (tags) and strategically placed acoustic receivers has been successfully used to monitor the movements of fish, particularly salmonids, within a number of river catchments and associated estuaries in the UK (Moore et al., 1995; Bendall et al., 2005; Bendall & Moore 2008; Moore et al. 2008). The same technique has also been recently used to study the behaviour of silver eels in estuaries and fjords in Europe and Scandinavia (Aarestrup et al., 2008, 2010; Davidsen et al., 2011).

The acoustic transmitters and receivers (Figure 2.7) that were used in the present study were manufactured by VEMCO (Canada).

Figure 2.7 Vemco acoustic receiver and V9 tags as used in the eel tracking studies.

Eels were tagged with model V9T-1x-69KHz transmitters (9 mm in diameter x 39 mm in length, weighing 5.3 g) incorporating a temperature sensor (range 0-40°C) and

operating at 69 kHz. The acoustic receivers deployed to detect the eels as they migrated out to sea were the VR2W model.

The V9 transmitters were surgically implanted into the peritoneal cavity of the eels using a technique described by Moore et al. (1990). The use of surgical implantation techniques to attach telemetry tags to fish has been carried out in the UK since the late 1980s (Lucas, 1989; Moore et al., 1990). The technique is particularly useful on eels as external attachment of a tag is likely to increase drag during swimming with the associated metabolic cost and potential to cause injury and infection to the free swimming fish at the point of attachment (Cooke et al., 2011). The eels were anaesthetised with a solution of 2- phenoxyethanol, (concentration 2 ml l^{-1} in water) to allow surgical implantation. After the fish was fully anaesthetised it was placed into a V-shaped tagging holder and an incision 10-13 mm in length was made in the abdomen using a sterile disposable scalpel 3-4 cm anterior to the anal opening. The tag was then inserted into the body cavity and the incision closed with two absorbable Vicryl sutures (Ethicon). The sutured incision was then treated with a 50:50 mixture of powered antibiotic (amoxicillin) and Orahesive which allowed the antibiotic to adhere to the wound. Previous studies on other fish species have shown that the incision rapidly heals and that the sutures are lost within 2-3 weeks (Moore et al., 1990).

The eels were then weighed (g) and measured to the nearest mm from the snout to the end of the tail. The eels were then moved to an aerated tank of water and allowed to fully recover from the anaesthetic and handling. Recovery was judged by the full ability to orientate and swim. After recovery the eels were returned to a holding/experimental tank before being released close to their point of capture in the river 5-15 days later.

The acoustic receivers used to detect the subsequent movements of the released eels were deployed at strategic positions throughout the river and estuary to monitor the eels as they migrated seawards. Receivers were normally deployed at positions such as the head of tide, saline limit and exit of the estuary. The limit of saline incursion and tidal influence in an estuary are not synonymous, normally the influence of the tides extends further upstream than the salinity limit (Adam, 1990). The receivers were attached to existing structures in the river and estuary such as jettys, channel marker buoys or tethered to trees adjacent to the water course. Each tag transmitted a unique

ID code followed by the temperature data at variable periods between 20 and 60 s. When in range of a receiver (~300 m), this information together with the date and time of detection was recorded and logged in the receiver's memory and later downloaded to a laptop PC. Downloads were undertaken at periods between 4 and 10 weeks. Subsequent analysis of the data provided a chronological record of detections for each tag at each receiver location. From this information a 'track' of previous positions of individual fish was generated and the movements of the fish as they migrated out to sea could be compiled. The movements of the eel were related to diurnal activity, tidal activity and speed of movement over the ground. The downstream migration of tagged eels as they were initially detected by each of the acoustic receivers were analysed using circular statistical methods, testing whether the movement of eels was random with respect to time of day and state of tide using the Raleigh test ('*r*' value) (Batschelet, 1981). High water at the head of tide in each river system has been used as a reference time for all tidal cycles. The differences in migration times of the exposed and control groups of eels within each section of the river and estuary have been compared using a simple t-test or a Mann-Whitney Rank Sum Test.

Chapter 3. Juvenile stage

3.1. Introduction - transition from the marine to the freshwater environment

Once eel larvae complete their transoceanic migration and reach the continental shelf they metamorphose into glass eels, move towards estuaries where they further develop into pigmented elvers (Crean et al., 2005) and subsequently colonize coastal and inland waters (Gascuel, 1986). The movement within the immediate coastal zone and through the estuarine environment is completed using selective tidal stream transport (McCleave & Wippelhauser, 1987). During flood tide the eels swim up into the water column and orientate towards the mouth of rivers following olfactory cues emanating from the river (Sorensen, 1986; Sola, 1995) and are subsequently transported landwards by the prevailing tidal currents. During slack water, they move down to the substratum and remain close to the bottom until the following flood tide when they continue their migration towards fresh water. The migration of the glass eel is therefore considered to be through saltatory steps (Edeline et al., 2005b). However, not all glass eel will enter the freshwater environment. A portion of the population will settle in the estuarine and coastal environment (Tzeng et al., 1997, 2002; Tsukamoto & Arai 2001; Jessop et al., 2004) while others will move frequently between the freshwater and marine environment (Feunteun et al., 2003). Various authors have suggested that once glass eel get close to freshwater, they need a period of acclimation before entering freshwater in order to go through physiological and behavioural changes which will allow them to successfully adapt to freshwater (Deelder 1952; Sorensen & Bianchini, 1986). However, in a number of experimental studies glass eels have been shown to survive acute salinity challenges when transferred directly between saltwater and freshwater (Wilson et al., 2004; Crean et al., 2005). A further study carried out on glass eels entering the River Guadalquivir in Spain (Arribas et al., 2012) suggested that those physiological and behavioural changes occur gradually along the salinity gradient in the estuary. Crean et al., (2005) found that even though glass eels always survived abrupt changes in salinity, having developed into fully pigmented elvers they do suffer mortality if transported back to full strength seawater.

This suggests that the physiological changes that the elvers have incurred in preparation for fresh water entry may not be rapidly reversed.

During the development from glass eel to the pigmented elver stage within the estuarine environment, individuals may be exposed to a wide range of diffuse and point source contaminants derived from various sources. These may have a deleterious impact on the initiation and successful transition from the saline to the fresh water environment.

The potential impact of contaminants on this transition between environments is investigated within this chapter. The Chapter details a number of laboratory-based experiments examining how exposure to specific, environmentally relevant contaminants may affect key physiological processes that are involved in and necessary to successful freshwater adaptation (e.g. gill ATPase activity) and the subsequent survival of the eels.

3.2. Glass eel and tributyl phosphate

3.2.1. Methods

In April 2010, ~500 glass eels (Figure 3.1) were collected from Glass Eel UK and transported to Cefas Lowestoft laboratory as described in Section 2.1.

Figure 3.1 Glass eels.

Once in the laboratory, eels were distributed equally between six identical 63 l glass tanks (85 eels in each tank) and supplied with brackish water (average salinity 18 ppm) at ambient temperature (ranging between 10 and 12°C). Each tank was gently aerated and enriched with hiding features and three sides and the top were blacked out to create an environment more suitable for glass eels.

Fish were left to acclimatize for ten days. At the end of acclimation three of the six tanks were exposed in flow through conditions to 0.5 $\mu g\ l^{-1}$ of tributyl phosphate (TBP) while the other three tanks were used as controls. Fish were exposed for two weeks in a flow-through environment as described in Section 2.4. Fish were not fed during the experiment. At the end of the two-week exposure period, dosing with TBP was discontinued and half of the fish from each tank were sampled. The remaining fish underwent a freshwater challenge test. During the freshwater challenge test the water inflow to all tanks was changed from brackish to freshwater and the fish were monitored daily for a period of three days. At the end of the three days, freshwater challenge test, all remaining fish were sampled. To allow for logistic restriction in sampling large number of fish, the dosing was staggered and commenced in subsequent days (one control and one exposed tank per day over 3 days). Fish were sampled according to the methods described in Section 2.2. To allow for enough sampling tissue for Na^+/K^+ATPase, gills of ten individuals were pooled into one sample.

Water samples were collected at the end of the experiment and sent for analysis to determine actual concentration of TBP. Analysis was carried out in the Chemistry department of Cefas Lowestoft Laboratory. Samples were analysed by gas chromatography and the results indicate non detectable concentration of TBP in the control tank and a concentration of 0.25 $\mu g\ l^{-1}$ in the exposed tanks (average between the concentrations measured in the three replicates).

Tributyl phosphate (TBP, CAS Nr 126-73-8) is an organophosphate ester (Figure 3.2) which is a class of chemicals also widely used as a flame retardant or plasticiser in a number of commercial products (Campone et al., 2010).

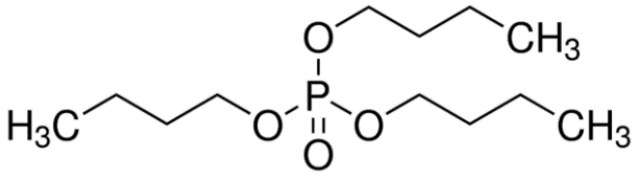


Figure 3.2 Tributyl phosphate chemical structure.

TBP is used as a solvent, plasticizer, antifoaming agent, metal extractant and flame retardant. When used in industrial processes as a solvent, extractant or antifoaming agent it is constantly lost to the air and aquatic environment (WHO Report, 1991). Its biodegradation is slow and its concentration in water is not decreased by standard techniques used in drinking water treatments. Concentrations measured in water range from nanograms to 52 μg l^{-1} (WHO, 1991; Marklund et al., 2005). The solubility of TBP in water at ambient temperature is less than 1 g l^{-1} and the difference in concentration between water and sediment is ~ 3 orders of magnitude. The 96 hrs LC_{50} for fish is in the range of 4.2-11.4 mg l^{-1} (WHO, 1991) but there is a poor understanding of TBPs mechanistic effects on fish although it is known to directly affect the kidney in rats and mice (Oishi et al., 1982; Laham et al., 1985) an important organ in controlling osmoregulation in fish.

3.2.2. Results and discussion

The results of the experiment indicated that there was no difference in mortality between the control groups and exposed fish and no difference was observed between treatments in terms of the levels of gill Na^+/K^+ ATPase activity (Figure 3.3). However, fish did differ in their condition factor (CF) (Figure 3.4) with control fish sampled at the end of the exposure period in brackish water having a lower CF than all other groups.

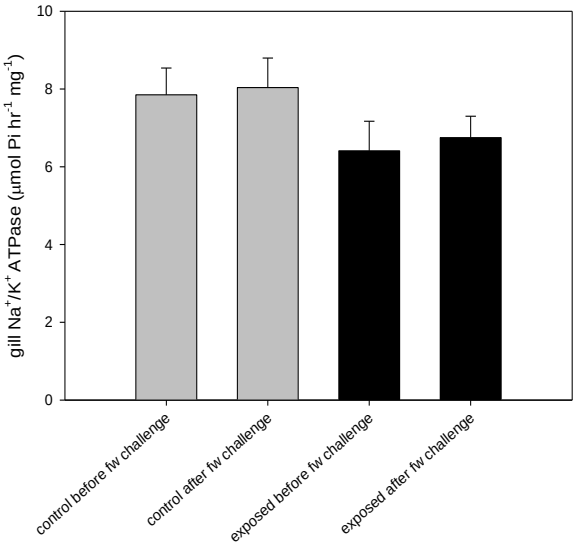


Figure 3.3 Gill Na+/K+ ATPase levels in glass eels exposed to tributyl phosphate. Bars represent mean ± standard error. 2-way ANOVA, General Linear Model, between treatments P=0.058, before and after freshwater challenge P=0.705, interaction P=0.914.

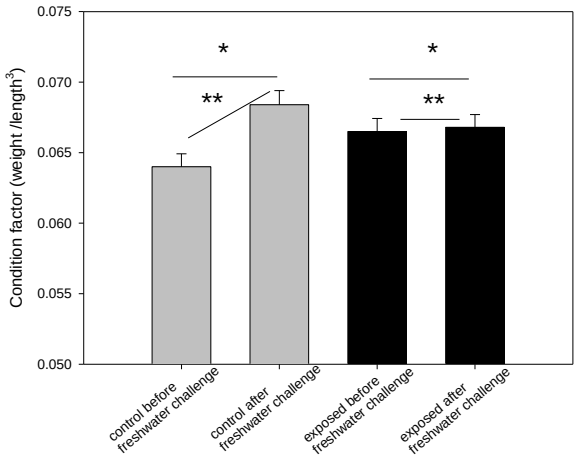


Figure 3.4 Condition factor of glass eels exposed to tributyl phosphate. Bars represent mean ± standard error. Sample size n=120 (each control and exposed before freshawater challenge), n=101 (control after freshwater challenge), n=127 (exposed after freshwater challenge). 2-way ANOVA, General Linear Model, between treatments P=0.603, before and after freshwater challenge P=0.013, interaction P=0.028.

Considering the short duration of the exposure period, this could be due to an unequal distribution of fish sizes across the tanks at the beginning of the experiment. However, this is probably unlikely as the sample size for this experiment was quite high (40 fish per treatment sampled at the end of the exposure period and 32 to 44 fish per treatment sampled after freshawater challenge) and the fish were allocated to the various tanks (3 replicates for each treatment) randomly on arrival as they were all of very similar size and at the same developmental stage. This observed difference in CF may indicate a differential allocation of resources of the fish held in different water quality. A correlation between fish CF and contaminant exposure has been shown for various fish species. A study on red-ear sunfish (*Lepomis microlophus*) indicated that chronic, low-level selenium exposure was linked to fish showing accumulation of selenium in various organs and reduced condition factor (Sorensen & Bauer, 1984). The authors suggest that the reduction in CF could be due to selenium induced toxicity such as anoxia, poor food conversion and stunting. Another study investigating the effect of selenium found that in juvenile bluegill (*Lepomis macrochirus*) elevated selenium caused haematological changes and gill damage that reduced respiratory capacity. However only when selenium exposure was accompanied by a decrease in water temperature the CF of the fish was negatively affected (Lemly, 1993). Two studies on the effect of bleached kraft mill effluent on white suckers (*Catostomus commersoni*, Hodson et al., 1992) and redbreast sunfish (*Lepomis auritus,* Adams et al., 1996) showed opposite results, with white suckers downstream of the pollution source showing a decreased CF, while the sunfish immediately downstream of the pollution source showed the highest CF that was gradually returning to the same level as for fish present upstream of the mill the further downstream the fish were sampled. The differences found between these studies could possibly reflect site-specific differences in energy allocation strategies and may also be related to other environmental factors other than contaminant exposure (Adams et al., 1996). It is difficult to draw strong conclusions from the study presented here as no other physiological parameters were available from the experimental fish mainly due to their small size. For example, due to the small size of the individuals it was not possible to collect blood samples for plasma ion analysis.

3.3. Glass eels and metals

3.3.1. Methods

In April 2013, ~ 1000 glass eels were collected from Glass Eel UK and transported to the Cefas Lowestoft Laboratory. Fish were maintained in sea water in a 500 l tank at ambient temperature and with naturally simulated photoperiod until the beginning of the experiment. Fish were fed daily (Monday to Friday) with commercial fish pellets of appropriate size (Skretting, UK). A week before the start of the experiment, 30 glass eels were transferred to one of 22 glass aquaria (50 l) with running seawater. The tanks were placed side to side in two rows of 11. The sides and back of all tanks were blacked out to prevent visual contact and disturbance between adjacent tanks. All tanks were provided with three hiding features (dark ceramic pots) and an airline providing constant aeration. When the experiment started the water supply to the tanks was stopped and the fish were kept in static, aerated seawater. The tanks were then dosed with one of the ten metal treatments shown in Table 3.1 each with a replicate. A control tank with a duplicate was also included in the experiment where the fish were placed in natural seawater.

Table 3.1 Treatments used for water borne exposure of glass eels.

Treatment	**Concentration (µg l^{-1})**	**Treatment**	**Concentration (µg l^{-1})**
Copper low (Cu low)	2	Copper high (Cu high)	10
Lead low (Pb low)	1	Lead high (Pb high)	5
Zinc low (Zn low)	5	Zinc high (Zn high)	20
Chromium low (Cr low)	1	Chromium (Cr high)	4
Mixture low (Mix low)	2 (Cu), 1 (Pb), 5 (Zn), 1 (Cr)	Mixture high (Mix high)	10 (Cu), 5 (Pb), 20 (Zn), 4 (Cr)

The metals used for the experiment were copper sulphate ($CuSO_4$ $5H_2O$, CAS Nr 7758-99-8) for copper; lead nitrate ($Pb(NO_3)_2$, CAS Nr 10099-74-8) for lead; zinc sulphate ($ZnSO_4$ $7H_2O$, CAS Nr 7446-20-0) for zinc and potassium chromate (K_2CrO_4, CAS Nr 7789-00-6) for chromium.

Half of the water within each tank was replaced with freshly spiked seawater every three days. Fish were no longer fed once the dosing experiment started. To allow for logistic restriction in sampling large number of fish during the same day, the experiment was staggered with each of four tanks at a time being dosed over a period of four days and the remaining six tanks on the fifth day. Sampling was carried out in the same order so that all fish experienced exactly the same length of exposure. Glass eels were kept in the experimental tanks for two weeks. Behavioural observations were taken daily as described in Section 2.5 to look for changes in activity level between the different treatments. After the two-week exposure, ten fish were sampled from each tank for morphological and physiological parameters. In addition, a blood sample was also taken for DNA investigation. The blood samples were used to carry out a Comet assay as described in Chapter 2. The remaining 20 fish in each tank then underwent a freshwater challenge test for 3 days in clean, flow through dechlorinated tap water. After the 3-day test, eels were sampled for morphological and physiological parameters.

Metals were chosen for this experiment as they are one of the major contaminants present in the Severn estuary and Bristol Channel where most of the glass eels entering UK inland waters are found. The low and high concentrations were chosen to represent historic values measured during 1970's (high concentration groups) when the glass eel recruitment decline started and more recent measured concentrations (low concentration groups) as metal pollution of the Severn estuary and Bristol Channel has decreased over the last few decades.

The Severn estuary is the largest estuary in the UK and the River Severn Catchment covers over 10 000 km^2. The catchment drains a large part of the English Midlands, which includes large urban centres and mid-Wales, which is predominantly rural in character (Aprahamian et al., 2007). The main river flows 350 km to its estuary downstream of Gloucester, where it widens into the Bristol Channel (Figure 3.5). The funnel shape of the estuary, the high tidal range (13.2 m mean spring) and its south-westerly orientation all combine to promote strong recruitment of glass eels (Aprahamian et al., 2007) which typically occur in large runs during spring (White & Knights, 1997).

Figure 3.5 The Bristol Channel.

For several decades the Severn Estuary and the Bristol Channel have been contaminated by heavy metals originating from diverse sources. An important source of metals are the various sewage treatments works that service the large cities of Bristol, Gloucester, Newport, Cardiff and Swansea. Historically several mining activities were concentrated around the Bristol Channel but they have now been closed or reduced. In addition, several chemical industries are situated around Avonmouth (close to Bristol) which contribute to the metal discharge in the estuary (Duquesne et al., 2006).

Data on the trends in concentrations of dissolved and sediment bound metals for the Severn Estuary are presented in tables 3.2 and 3.3, while table 3.4 shows the concentrations of metals detected in worms collected from the Bristol Channel. These data have been used to estimate the concentrations of metals eels have been likely exposed to in the past and present times via water or sediment or prey consumption.

Table 3.2 Dissolved metal concentration (µg l^{-1}) in the Severn Estuary. Adapted from Jonas & Millward, 2010.

Year	Cu	Pb	Zn	Cr	Cd	Ni	Ref
1971	1.49-2.24	1.73-1.38	10.5-8.13		1.38-1.94	0.99-1.11	Abdullah &Royle 1974
1975-1979	2.2-4.2	1.5-4.1	11-22		0.31-1.48	1.9-3.6	Owens 1984
1984	1.5-4.5	1.5-3	10-23		0.3-3	1.5-3.5	Morris 1984
1987	1.7-4.7	0.02-10			0.01-0.14		Harper 1991
1988	2.15±1.17		0.5±17.5	0.23-0.95	0.03-0.45	0.1-16	Apte et al 1990a,b
2004	2.58±0.86	<2.5	5.67±1.67	1.02±0.67	<0.25	<4	Jonas & Millward 2010
2005	3.08±2.14	<2.5	7.02±4.14	1.26±0.94	<0.25	<4	Jonas & Millward 2010

Table 3.3 Metal concentration (µg g^{-1}) in the sediment of the Severn Estuary. Adapted from Jonas & Millward, 2010.

Year	Cu	Pb	Zn	Cr	Cd	Ref
1971	157-261	163-159	362-520		9.7-19.1	Abdullah &Royle 1974
1979	55.4	193	461	335	2.61	Hamilton et al 1979
2004	3.6-56		60-907	16.8-340		Jonas & Millward 2010
2005	0.5-168		76-403	6.3-37.5		Jonas & Millward 2010
2006	30-37	65-78	191-238	67-81	0.32-0.41	Duquesne et al 2006

Table 3.4 Metal concentration (mg (kg dry weight)$^{-1}$) in worms (*Hediste diversicolor*) from the Severn Estuary (from Langston et al., 2010).

Year	Cu	Pb	Zn	Cr	Cd	Ni
1978	46.9±5.3	2.87±0.18	250±9.98	0.19±0.02	3.47±0.39	5.22±0.26
2004	76.5±7.59	1.92±0.18	205±11.3	2.11±0.3	0.59±0.07	2.64±0.2
2005	59.7±9.18	1.75±0.17	192±15.5	1.8±0.25	0.43±0.05	2.19±0.19

3.3.2. Results and discussion

Water samples were taken after one week and at the end of the 2-week exposure and sent to the National Laboratory Service for analysis of actual metal content. Results of the water analysis are presented in Table 3.5.

Table 3.5 Results of water analysis carried out by National Laboratory Service.

Tank	Treatment	expected conc µg l^{-1}	copper µg l^{-1}	lead µg l^{-1}	zinc µg l^{-1}	chromium µg l^{-1}
1- halfway	mix low	2 - 1 - 5 - 1	5.14	0.158	4.4	0.52
1-end	mix low	2 - 1 - 5 - 1	3.61	0.214	2.74	<0.5
2-halfway	mix high	10 - 5 - 20 - 4	16.7	0.74	6.73	1.57
2-end	mix high	10 - 5 - 20 - 4	19.3	0.96	5.16	1.4
3-halfway	Cu low	2	6.51	0.129	4.19	<0.5
3-end	Cu low	2	3.97	<0.04	2.29	<0.5
4-halfway	Cu high	10	7.31	0.052	8.81	<0.5
4-end	Cu high	10	6.12	0.071	4.63	<0.5
5-halfway	Pb low	1	7.31	0.142	3.71	<0.5
5-end	Pb low	1	2.43	0.097	1.6	<0.5
6-halway	Pb high	5	5.38	0.446	4.08	<0.5
6-end	Pb high	5	2.69	0.651	2.05	<0.5
7-halfway	Zn low	5	6.28	<0.04	149	<0.5
7-end	Zn low	5	1.93	<0.04	11.3	<0.5
8-halfway	Zn high	20	4.17	<0.04	7.71	<0.5
8-end	Zn high	20	2.22	<0.04	5.4	<0.5
9-halfway	Cr low	1	3.84	<0.04	2.56	<0.5
9-end	Cr low	1	1.98	<0.04	1.92	<0.5
10-halway	Cr high	4	3.33	0.047	3.15	1.29
10-end	Cr high	4	2.28	0.05	3.19	1.29
11-halway	Control		5.03	0.054	7.91	<0.5
11-end	Control		2.15	<0.04	3.83	<0.5

The measured concentrations of the metals in the study were predominantly lower than the expected concentrations (see Table 3.5). This is often the case in toxicological studies where there can be uptake of the metals onto the tank and possible settling of the heavier metals. In this study, all metals showed this trend except zinc at low

concentration where the measured concentrations were higher than expected (much higher on the first sampling occasion). The results from the zinc exposure should therefore be considered with caution as this may have been due to contamination of the samples either during the experiment or after collection and during analysis.

The results of the experiment indicated that there were no significant differences (Table 3.6) in the measured morphological and physiological parameters (Tables 3.7 and 3.8) between the various metal treatments and control. The behavioural observations were taken daily during 10 s snapshots. Very few fish were ever seen active and therefore the data were not sufficient for statistical analysis.

Table 3.6 Statistical analysis. 2-way ANOVA, General linear model.

	Differences between		
	Treatments	Salt vs freshwater	Interaction
Weight	0.055	0.036	0.018
Length	0.301	0.736	0.140
CF	0.050	0.059	0.106
ATPase	0.663	0.167	0.127

Pairwise multiple comparison (Holm-Sidak method) indicated no significant difference between the various treatments and control, but the only significant difference in weight and CF was between Cr low and Cu high.

Table 3.7 Morphological and physiological data from eels exposed to metals in seawater.

Before freshwater challenge								
treatment	weight (g)		length (mm)		Condition Factor		ATPase (μmol Pi (mg hr)$^{-1}$)	
	mean	Sem	mean	sem	mean	sem	mean	sem
control	0.16	0.012	68.34	0.74	0.051	0.003	5.89	2.69
Cu low	0.13	0.011	66.25	0.94	0.043	0.003	9.72	1.53
Cu high	0.11	0.007	66.11	0.53	0.036	0.003	7.23	2.21
Pb low	0.15	0.010	67.96	0.79	0.045	0.002	9.87	0.93
Pb high	0.13	0.008	67.01	0.82	0.044	0.002	12.71	1.42
Zn low	0.12	0.010	66.82	0.77	0.040	0.003	8.38	1.72
Zn high	0.14	0.012	67.28	0.77	0.044	0.003	10.69	0.99
Cr low	0.20	0.051	68.65	0.67	0.060	0.015	9.29	2.16
Cr high	0.13	0.008	66.50	0.63	0.044	0.002	9.68	1.51
mix low	0.13	0.009	66.91	0.39	0.044	0.003	10.99	2.08
mix high	0.16	0.012	69.43	0.79	0.047	0.002	7.47	1.14

Table 3.8 Morphological and physiological data from eels transferred to clean freshwater after chemical exposure in seawater.

After freshawater challenge								
treatment	weight (g)		length (mm)		Condition Factor		ATPase (μmol Pi (mg hr)$^{-1}$)	
	mean	Sem	mean	sem	mean	sem	mean	sem
control	0.12	0.007	66.93	0.77	0.039	0.002	11.18	1.21
Cu low	0.13	0.007	67.23	0.51	0.044	0.002	6.97	1.71
Cu high	0.13	0.010	67.37	0.76	0.040	0.002	8.44	2.02
Pb low	0.13	0.008	67.78	0.59	0.040	0.003	9.59	0.91
Pb high	0.13	0.008	68.05	0.61	0.040	0.002	7.59	1.59
Zn low	0.13	0.008	67.59	0.53	0.042	0.002	8.01	2.57
Zn high	0.12	0.007	67.28	0.51	0.040	0.002	11.54	3.45
Cr low	0.13	0.007	67.09	0.42	0.044	0.002	8.04	1.40
Cr high	0.15	0.007	68.52	0.52	0.045	0.002	7.98	0.87
mix low	0.13	0.006	66.28	0.63	0.045	0.001	4.86	2.69
mix high	0.14	0.006	66.62	0.61	0.046	0.002	6.73	1.56

The result of the Comet assay (Figure 3.6) indicated that the mean tail moment (i.e. DNA damage) was significantly higher in exposed eels compared to the control eels (one-way ANOVA, Kruskall-Wallis, H=121.191, 10 degrees of freedom, P<0.001. Multiple comparisons against control, Dunn's method P < 0.05).

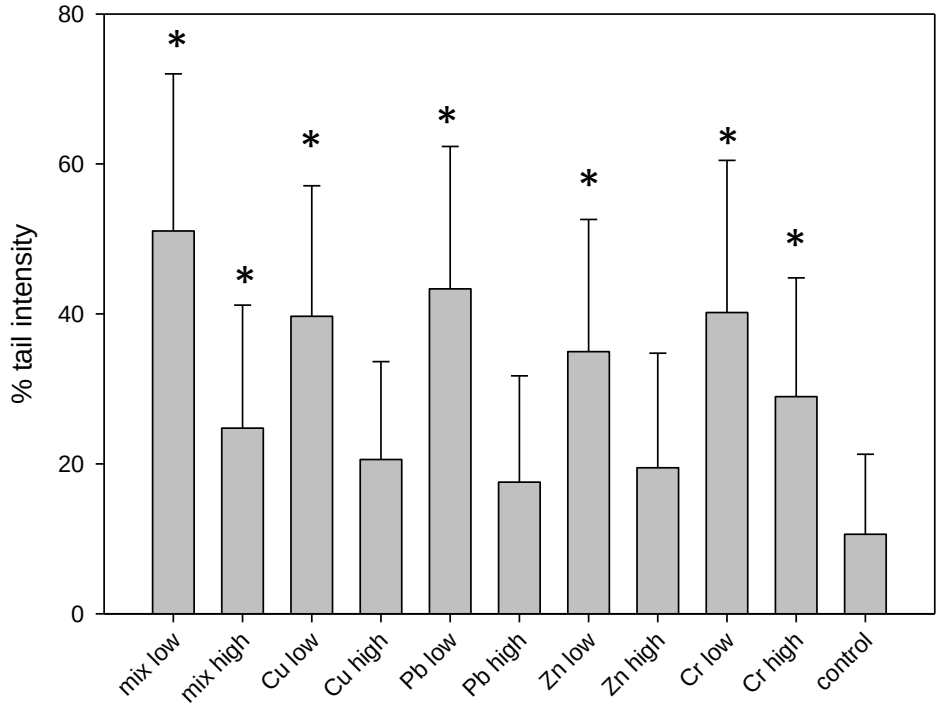


Figure 3.6 Comet assay results of glass eels exposed to metals in seawater for 2 weeks. The graph shows the mean tail intensity (and standard deviation) which represents the level of DNA damage sustained by the fish. Results for the 10 fish in each treatment tanks and for the 2 duplicate tanks of each treatment are combined to give an average per treatment.

All the eels exposed to the low concentrations of the metals demonstrated significant damage to the DNA. However, in the high concentration metal groups it was only the mixture and Cr groups that showed a significant damage to the DNA. The results indicate that there was greater damage to the DNA by lower doses of the mixture of metals, copper, lead and chromium than at the higher dose. It is generally assumed in toxicology that the dose-response curve is monotonic, i.e above a certain concentration

(a “threshold”) increasing dose leads to increasing response. However, regarding some contaminants and in particular endocrine disrupting chemicals, non-monotonic dose response curves i.e. bell shaped or (inverted) U-shaped dose response relationships are increasingly being described (see Vandenberg et al., 2012; Rhomberg & Goodman, 2012). This appears to be the case in this study on metal exposure where lower doses produce more damage to the DNA than the higher doses. However, the exact mechanisms for how the metals caused damage to the DNA are not known. DNA damage can be induced through apoptosis or necrosis, secondarily through the interaction with oxygen radicals or other reactive intermediates, or as a consequence of exclusion repair enzymes. The metals could be acting as a chemical stressor that induces oxidative stress as it has been shown for water borne selenium in juvenile rainbow trout (Miller et al., 2007). Although there was damage to the DNA of the eels, it is not known what the long term effect of this would be to the viability and survival of the eels.

Chapter 4. Growing stage

4.1. Introduction

Research undertaken in a previous Defra funded collaborative project between Cefas Lowestoft Laboratory and the University of Portsmouth (Crooks, 2011 - Defra Project SF0240) has indicated that survival of the intragravel stages of salmonids is affected by the environmental levels of both sediment-bound and water borne contaminants circulating within the gravels.

During their initial residence in freshwater, juvenile eels are often buried for long periods within the river sediment during the day and as a result will be directly exposed to both sediment as well as water borne contaminants.

In addition, their diet will consist of prey items also present in the substratum, which may provide another potential route for uptake and bioaccumulation of toxicants. This chapter describes laboratory-based experiments to investigate the potential impact of contaminants on the eels during the early freshwater stage where exposure may influence both growth and subsequent survival. It further investigates the effect of contaminant exposure on eel olfaction as this is considered to be a very important sense for eels in prey detection, orientation, migration and reproduction (Tesch, 2003).

This Chapter identifies the toxic compounds commonly occurring in river sediments and examined their impact on juvenile eels. Experiments were undertaken where groups of juvenile eels were exposed to environmentally relevant concentrations of key contaminants bound to sediment, and their impact on the development and survival of individuals was quantified. Experiments were also undertaken to assess the effect of contaminated food on growth and survival. Additional groups of eels were fed food items spiked with known concentrations of contaminants and their survival subsequently monitored. Tissue samples from each group were analysed to determine the potential impact on DNA integrity and gene expression.

4.2. Elvers and Hexabromocyclododecane

4.2.1. Methods

In May 2011, 70 glass eels were collected from the River Tees and transported to Cefas Lowestoft Laboratory where they were kept in a large 500 l tank to acclimatise. The tank was supplied with constant ambient freshwater (temperature range 15-16 °C), an airline and a naturally simulated photoperiod (15:9 hours of light:dark). Fish were fed daily (Monday to Friday) with commercially available brine shrimp (*Artemia spp.*). In June the fish were equally distributed over 4 glass tanks (16 fish per tank with volume of 63 litres) containing each 5 kg of fine gravel and supplied with running freshwater at ambient temperature (16-19.5 °C). The eels were left to acclimatize for ten days and then half of the fish from each tank (seven fish per tank as one fish had died in two of the experimental tanks) were collected, measured and cryo-preserved for subsequent analysis. The remaining animals were presented with weekly addition of clean or spiked clay (Newplast™ clay modelling clay - Newclay Products Ltd, Newton Abbot, UK) for a period of three months to simulate the cumulative effect of regular run offs. Each of the treatments was done in duplicate tanks. Prior to use, the clay was combusted in an oven at 450ºC for five hours to remove any organics (Crooks, 2011). It was then mechanically ground with a mortar and pestle to achieve a fine sediment particle size. Toxicity information supplied by Newclay Products Ltd and measured under BS EN 71-3, indicated undetectable levels of common metals (e.g., mercury, lead, cadmium, copper, arsenic) in the modelling clay. To prepare the clay for the exposure experiment, for each tank, 10g of fine clay was measured and for the exposed tanks it was spiked with 5 ml of a solution of hexabromocyclododecane (HBCD, CAS Nr 3194-55-6) dissolved in acetone at a concentration of 500 µg ml^{-1} in order to achieve the desired concentration of 500 µg kg^{-1} in the tank containing 5 kg of sediment. The clay for the control tanks was spiked with 5 ml of acetone. All spiked clay was left for at least 5 hours in an exhaust ventilation unit to completely dry before being added to the tanks. The concentration of the BFR added on a weekly basis was chosen to mimic potential run-off from land alongside natural watercourses. The concentration of HBCD was consistent with values described in published literature and equal to 500 µg kg^{-1} of sediment (Morris

et al., 2004). At the end of this period, all surviving eels were sampled, anaesthetized then measured for length and weight and cryo-preserved for further analysis.

HBCD is used as a flame retardant. These are substances used in the manufacture of a wide range of materials such as plastics and textiles and are very prevalent in freshwater, estuarine and marine environments. The majority of flame retardants contain brominated organic compounds (Figure 4.1), making them persistent and lipophilic with the ability to bioaccumulate.

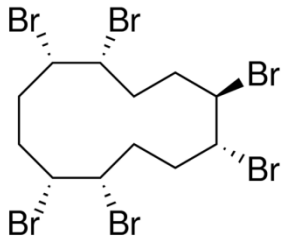


Figure 4.1 HBCD chemical structure.

HBCD is an additive flame retardant used principally in polystyrene foams but also in textile products (Marvin et al., 2011). It has a high bioaccumulation potential and is found in increasing levels in the environment and biota (Covaci et al., 2006; de Wit et al., 2010; Munschy et al., 2013). HBCD has been evaluated under the Stockholm Convention on Persistent Organic Pollutants and will be phased out between 2015 and 2019. Most brominated flame retardants are very similar in chemical structure to the thyroid hormones and they are likely to have an impact on thyroid function. Brominated flame retardants, have been shown to disrupt this endocrine system in many animals. In salmon, thyroid hormones play a vital role in smoltification and migratory behaviour (Boeuf, 1994; Iwata, 1995; Hutchinson & Iwata, 1998) and any modification of thyroid hormone concentrations (thyroxine T_4 and tri-iodothyronine T_3) are likely to significantly alter physiology and behaviour and may reduce the survival of smolts in the sea. Likewise in eels, thyroid hormones are involved in the metamorphosis of leptochephali to glass eels and have a regulatory function in pigment development, with higher concentrations of thyroid hormones resulting in higher rate of pigmentation (Jegstrup & Rosenkilde, 2003). Thyroid hormones are also involved in regulating glass eel behaviour with hormones levels being elevated while

the fish are in an actively swimming phase and lower concentrations are measured when fish show a more benthic behaviour (Jegstrup & Rosenkilde, 2003).

4.2.2. Results and discussion

The results of the study indicated there were no differences in either survival or the measured morphological parameters between control and exposed fish (Table 4.1). The eels' behaviour during the 3 month exposure was recorded by underwater cameras and saved on file, however the quality of the recordings was not sufficient to quantify any changes in behaviour and therefore no analysis was possible and it is not discussed further.

Table 4.1 Morphological parameters of elvers before and after 3 months exposure to 500 µg kg-1 of HBCD in the sediment. T-test between control and exposed at the beginning and end of experiment for length and weight was non-significant (P values reported).

	start (mean±sem)				**end** (mean±sem)			
	N	Length	weight	CF	N	length	weight	CF
control	14	67.67±1.19	0.29±0.02	0.09±0.005	14	79.58±1.98	0.53±0.06	0.09±0.007
exposed	14	70.58±1.75	0.34±0.06	0.09±0.007	8	84.65±3.08	0.74±0.13	0.11±0.006
t-test		0.16	0.43	0.66		0.16	0.11	0.12

Comparison for all the 3 measured variables between exposure treatment and start and end of experiment was carried out using a 2-way ANOVA (General Linear Model). All length, weight and CF were significantly different between the beginning and the end of the experiment ($P<0.001$) but there was no interaction with the treatment (for length $P=0.581$, weight $P=0.243$ and for CF $P=0.16$).

After considering the cost of carrying out further analysis and the likelihood of being able to detect statistically significant differences on such small sample size, no further analyses were carried out on the whole body of the glass eels that were cryopreserved. This was because the sample size at the end of the experiment was low (due to mortalities which occurred during acclimation and during the 3-month exposure

period) and the behavioural and morphological measurements indicated no differences between the treatments.

4.3. Elvers and metals

4.3.1. Methods

In June 2013, 500 juvenile eels (elvers) were transferred to the Cefas Lowestoft Laboratory as described in Section 2.1. Fish were kept in a large 500 l tank with flow-through ambient freshwater (temperature range 19-21.6°C) until the experiment started later in the year. The contaminants investigated were the same metals tested with glass eels in the experiment described in Section 3.3 and similarly a high concentration and a low concentration were tested. The concentrations tested are summarized in table 4.2 and were derived from studies presented in literature (see Tables 3.3 and 3.4 in Chapter 3).

Table 4.2 Concentrations of metals used in sediment and food exposure experiment.

treatment	Sediment (µg g^{-1})	Food (µg g^{-1})
control	None	None
Cu low	40	40
Cu high	80	80
Pb low	80	1.5
Pb high	120	3
Zn low	150	150
Zn high	300	300
Cr low	25	0.2
Cr high	50	2.5
Mix low	Cu 40; Pb 80; Zn 150; Cr 25	Cu 40; Pb 1.5; Zn 150; Cr 0.2
Mix high	Cu 80; Pb 120; Zn 300; Cr 50	Cu 80; Pb 3; Zn 300; Cr 2.5

At the beginning of the experiment 31 identical glass aquaria (50 l) were placed side by side along two rows (Figure 4.2). To avoid disturbance between tanks the sides and back of the tanks were blacked out with dark plastic sheets.

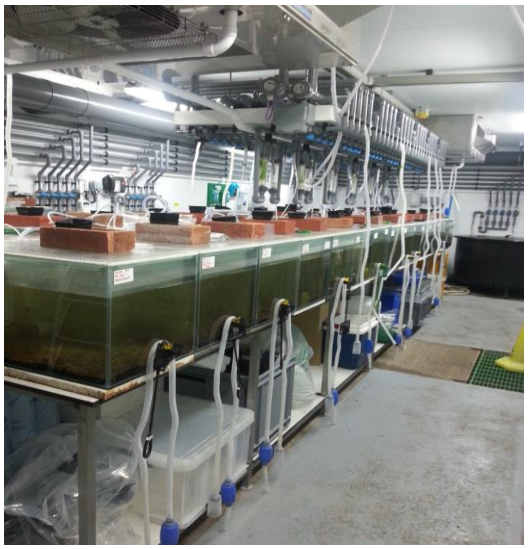


Figure 4.2 Aquaria set up for elver experiment.

Each tank was provided with 3 kg of artificial sediment made mixing 2 kg of pebbles (Velda classic pond bottom substrate) and 1 kg of soil (Velda Moerings pond plant soil) commercially available for use in ponds (Bradshaws direct, UK). After the batches of sediment were measured and mixed in a glass beaker, they were spiked with 200 ml of clean dechlorinated water or with 200 ml of the appropriate metal stock solution in dechlorinated water in order to achieve the concentrations indicated in table 4.2. After spiking, the sediments in the beakers were stirred and added to the bottom of their respective empty tank and allowed to air dry for a period of 24 hours before the start of the experiment. Each aquarium represented a different treatment where the metal tested would be present in the sediment or in the food or in both. Prior to the start of the experiment, the commercial food pellets (Labrax 2, Skretting – UK) were prepared (2 g per tank) and spiked with either 1 ml of de-ionized water or 1 ml of a water solution of the relevant metal and concentration for the treatment and left to dry for at least 4 hours. Tanks were filled with 50 l of ambient freshwater and an airline providing constant aeration. Between 10 and 20 fish were placed in each tank and the total wet weight of the fish placed in each tank was measured and recorded. The total weight rather than individual elver weight was taken to minimize the handling time

and avoid the use of anaesthesia at the beginning of the experiment. Single treatment tanks were used in this experiment as for logistic reasons a balance had to be found between testing all the possible combinations of metals and route of exposure and replication. The two main endpoints for this experiment were the Comet assay and gene expression of detoxifying genes and both those markers were thought to be very sensitive and able to detect differences between a relatively limited sample size.

Tanks were kept in static condition and fish were fed daily (Monday to Friday). Tank water was completely renewed every two days in all tanks.

To allow for logistic restrictions in sampling large number of fish, the experiment was commenced in four tanks per day over a period of seven days and sampling at the end of the experiment was carried out on four tanks per day to correspond to five weeks after the beginning of the experiment. Treatment had been allocated to tanks at random and the start of the test was also commenced in random order. Unfortunately, a problem occurred in the stock tank between the third and fourth days and some eels were lost. This was caused by a blockage of the tank outflow which resulted in the water in the tank overflowing allowing some animals to escape from the tank. As a result, the last ten tanks had gradually less fish allocated and one treatment (Cr High in sediment) had to be eliminated. The experiment lasted for 5 weeks at the end of which all fish in each tank were sampled for length and weight (Figure 4.3), a blood sample taken for Comet assay and the liver collected for gene expression determination.

Figure 4.3 Elvers being measured for weight and length.

4.3.2. Results and discussion

There was no difference in mortality between the various treatments. Table 4.3 indicates the morphological data measured at the end of the exposure experiment and the calculated weight increase from the beginning of the experiment. No statistical differences were observed in the weight (one-way ANOVA, Kruskal-Wallis, P=0.974), length (one-way ANOVA, Kruskal-Wallis, P=0.969), or calculated condition factor (one-way ANOVA, Kruskal-Wallis, P=0.172) between any of the treatments. The weight increase was obtained by the difference in the average individual weight for the tank at the beginning and at the end of the experiment as fish that had died during the experiment had been removed from the tank but not measured.

Table 4.3 Morphological data from elvers exposed to metals in either sediment, food or both.

			final weight		final length		CF		% weight
metal	conc	Medium	mean	sem	mean	sem	mean	sem	increase
control			1.74	0.31	103.65	5.05	0.132	0.004	17.42
Cu	low	Sed	1.95	0.39	106.80	5.93	0.125	0.005	23.54
Cu	low	Food	2.08	0.39	108.06	5.72	0.137	0.004	29.82
Cu	low	sed + food	0.93	0.05	89.80	1.91	0.128	0.005	37.63
Cu	high	Sed	1.61	0.31	102.38	5.66	0.127	0.008	23.83
Cu	high	Food	1.95	0.52	108.79	7.34	0.125	0.005	0.15
Cu	high	sed + food	1.71	0.28	104.25	5.88	0.127	0.007	27.05
Pb	low	Sed	1.90	0.37	107.71	6.75	0.127	0.004	32.57
Pb	low	Food	1.77	0.34	101.95	5.68	0.138	0.004	25.07
Pb	low	sed + food	1.59	0.26	99.74	4.52	0.136	0.007	23.54
Pb	high	Sed	1.74	0.26	104.77	4.77	0.130	0.003	22.68
Pb	high	Food	1.66	0.27	101.90	5.02	0.134	0.003	18.52
Pb	high	sed + food	2.53	0.51	114.07	8.59	0.139	0.003	35.13

Table 4.3 Continued

			Final weight		Final length		CF		% weight
metal	conc	Medium	mean	sem	mean	sem	mean	sem	increase
Zn	low	Sed	1.98	0.37	107.32	6.33	0.134	0.003	20.26
Zn	low	food	1.57	0.23	101.19	4.26	0.133	0.003	22.12
Zn	low	sed + food	2.62	0.78	111.63	9.32	0.128	0.005	28.38
Zn	high	Sed	1.82	0.25	104.94	4.54	0.142	0.003	33.78
Zn	high	food	0.93	0.11	88.78	2.49	0.128	0.006	41.94
Zn	high	sed + food	1.55	0.27	99.23	5.18	0.123	0.006	18.18
Cr	low	Sed	2.04	0.34	108.72	5.43	0.135	0.003	34.47
Cr	low	food	1.79	0.32	104.63	5.95	0.129	0.006	27.05
Cr	low	sed + food	1.49	0.27	100.15	5.04	0.121	0.004	8.87
Cr	high	food	2.01	0.43	107.26	6.54	0.128	0.004	21.71
Cr	high	sed + food	2.22	0.41	112.73	7.14	0.128	0.006	34.8
mix	low	Sed	1.69	0.25	103.80	5.18	0.135	0.003	34.91
mix	low	food	1.74	0.29	104.32	4.47	0.127	0.006	22.94
mix	low	sed + food	1.87	0.41	105.74	5.80	0.130	0.007	21.76
mix	high	Sed	1.86	0.34	104.70	5.76	0.133	0.004	25.46
mix	high	food	2.00	0.55	106.92	7.40	0.129	0.007	28.57
mix	high	sed + food	2.44	0.51	113.94	7.36	0.129	0.004	21.17

A sample of sediments from all tanks was collected at the end of the experiment and sent to the National Laboratory Service together with samples of the spiked food pellets for accurate analysis of metal concentration eels had been exposed during the test. Results of the sediment and food content analysis are presented in Tables 4.4 and 4.5. Highlighted in grey are the measured values for the metal that was added for the treatment.

Table 4.4 Food pellet concentration in μg g^{-1} as obtained from National Laboratory Service.

metal	Level	Expected conc	Copper	Lead	Zinc	Chromium
control		baseline	10.6	<1	174	0.748
Cu	Low	40	21.8	<1	175	0.875
Cu	High	80	33.9	<1	177	0.653
Pb	Low	1.5	10.5	1.47	175	0.712
Pb	High	3	10.3	2.92	175	0.696
Zn	Low	150	10.7	<1	212	0.951
Zn	High	300	11.5	<1	254	0.682
Cr	Low	0.2	10.9	<1	181	1.01
Cr	High	2.5	11.4	<1	180	1.7
Mix	Low	40,1.5,150,0.2	21.8	<1	212	0.823
Mix	High	80, 3, 300, 2.5	32.1	1.79	247	1.69

Table 4.5 Sediment concentration in μg g^{-1} as obtained from National Laboratory Service.

Metal	level	Expected conc	medium	Copper	Lead	Zinc	Chromium
control	control	baseline		34.2	6.36	65.2	23.1
Cu	Low	40	sediment	71.3	4.88	55.1	24.9
Cu	Low	baseline	food	26.7	4.4	60.3	25.8
Cu	Low	40	sed + food	94.5	4.64	52.7	12.1
Cu	High	80	sediment	161	5.85	77.2	23.7
Cu	High	baseline	food	32.2	7.35	61.8	25.3
Cu	High	80	sed + food	157	108	74.6	15.2
Pb	Low	80	sediment	28	292	62.5	16.6
Pb	Low	baseline	food	26.7	5.78	59.6	22.6
Pb	Low	80	sed + food	25.8	207	50.1	24.4
Pb	High	120	sediment	26.5	346	76.4	24.1
Pb	High	baseline	food	108	8.71	81.2	15.8
Pb	High	120	sed + food	31.1	382	65	23.5
Zn	Low	150	sediment	26.2	5.51	223	22.5
Zn	Low	baseline	food	24.3	4.43	52	24.7
Zn	Low	150	sed + food	34	6.32	281	14.4
Zn	High	300	sediment	24.4	4.57	316	25.5
Zn	High	baseline	food	34.2	5.26	72.6	19.2
Zn	High	300	sed + food	33.4	5.05	355	17.2
Cr	Low	25	sediment	27	5.08	54.9	62
Cr	Low	baseline	food	32.9	5.4	62.9	18.9
Cr	Low	25	sed + food	27	6.2	50.3	53.5
Cr	High	baseline	food	23.1	5.47	57.9	29.6
Cr	High	50	sed + food	31.2	7.53	54.6	84.3
Mix	Low	40, 80, 150, 25	sediment	90.1	252	211	51.8
Mix	Low	baseline	food	28.3	5.35	61.3	18.9
Mix	Low	40, 80, 150, 25	sed + food	95.6	247	359	49.7
Mix	High	80, 120, 300, 50	sediment	128	327	328	77
Mix	High	baseline	food	32.6	4.35	58.1	20.8
Mix	High	80, 120, 300, 50	sed + food	169	443	382	107

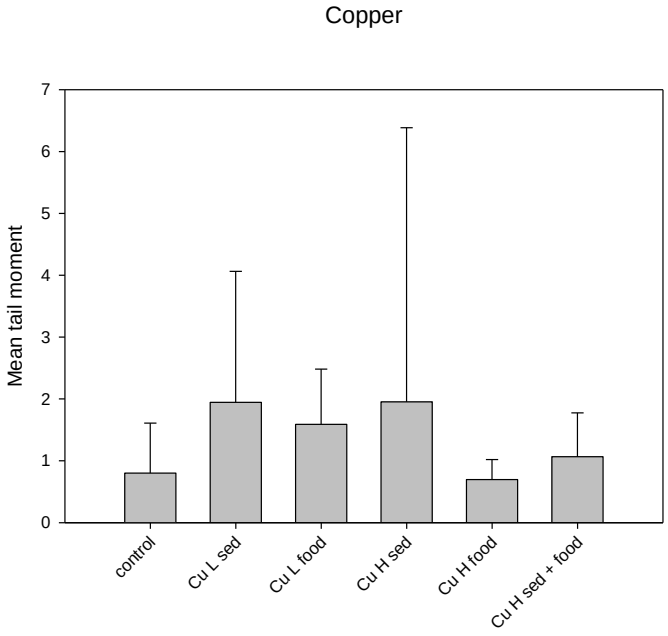
Comet assay results

Results of the comet assay are summarized in Table 4.6 and Figure 4.4 a to e present the mean tail moment for each metal tested against control.

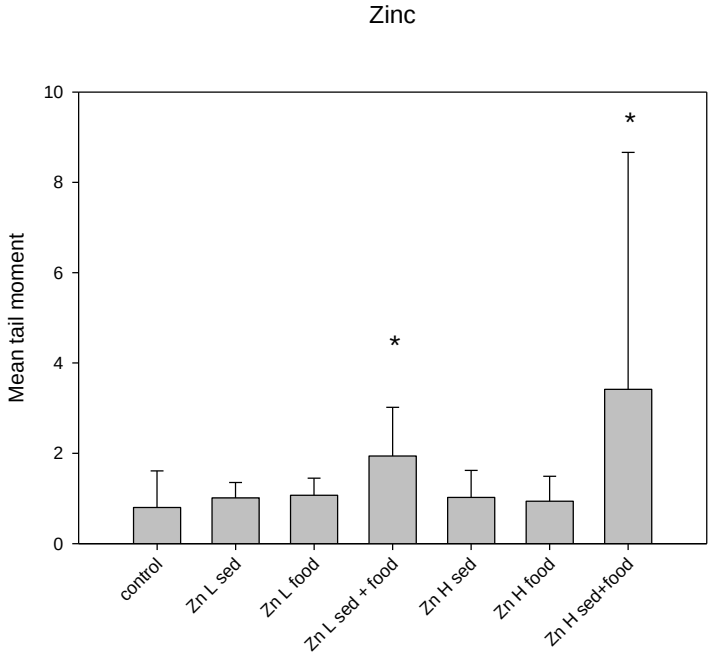
Table 4.6 Tail moment, standard deviation and sample size for each treatment.

Treatment	mean	st dev	N
Control	0.800	0.810	8
Cu l sed	1.945	2.118	10
Cu l food	1.590	0.892	10
Cu h sed	1.952	4.432	10
Cu h food	0.695	0.324	10
Cu h sed+food	1.065	0.709	10
Pb l sed	1.024	0.453	9
Pb l food	1.875	0.729	9
Pb l sed+food	0.742	0.249	5
Pb h sed	1.810	0.707	5
Pb h food	1.852	1.036	5
Pb h sed+food	0.680	0.592	10
Zn l sed	1.013	0.341	6
Zn l food	1.071	0.378	7
Zn l sed+food	1.941	1.079	9
Zn h sed	1.023	0.597	4
Zn h food	0.939	0.553	9
Zn h sed+food	3.416	5.248	10
Cr l sed	0.676	0.513	10
Cr l food	0.756	0.373	7
Cr l sed+food	0.409	0.285	10
Cr h food	0.605	0.302	10
Cr h sed+food	1.048	0.456	10
Mix l sed	1.293	0.471	10
Mix l food	0.369	0.257	10
Mix l sed+food	0.825	0.439	10
Mix h sed	0.711	0.322	10
Mix h food	1.260	0.719	10
Mix h sed+food	0.636	0.229	10

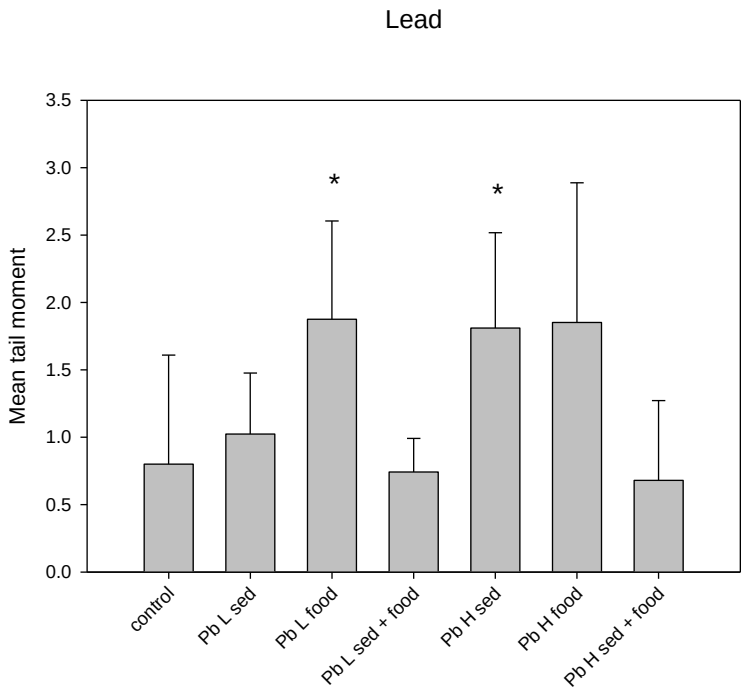
Figure 4.4 a-e. Juvenile eel DNA damage (Comet assay) in response to metal exposure via sediment and or food. The bars represent mean tail moment and the standard deviation. * indicate a statistically significant difference versus control (Multiple comparison, Dunn's method, P<0.05).



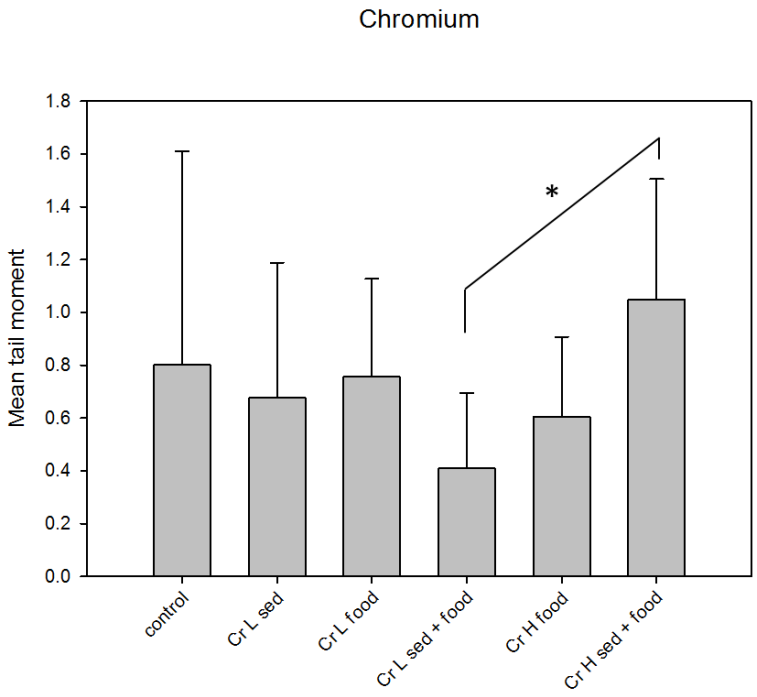
a. Copper, one-way ANOVA, Kruskal-Wallis: 5 d.f., P=0.014



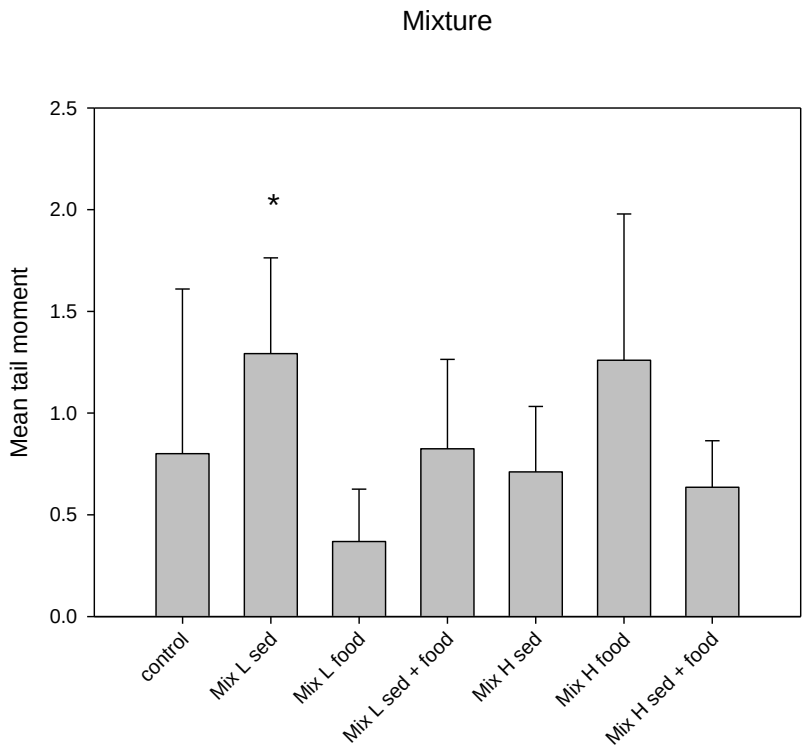
b. Zinc, one-way ANOVA, Kruskal-Wallis: 7 d.f., P=0.012. Multiple comparison versus control P<0.05 Zn low sed+food and Zn high sed+food



c. Lead, one-way ANOVA, Kruskal-Wallis: 6 d.f., P<0.001 Multiple comparison versus control P<0.05 Pb low food and Pb high sed.



d. Chromium, one-way ANOVA, Kruskal-Wallis: 5 d.f., P=0.020 Multiple comparison versus control P>0.05. Pairwise comparison P<0.05 Cr high sed+food VS Cr low sed+food



e. Mixture, one-way ANOVA, Kruskal-Wallis: 6 d.f., P<0.001 Multiple comparison versus control P<0.05 mix low sed.

Exposure to the metals in either the sediment or food had no significant effect on the measured morphological parameters in the elvers. Results of the Comet assay did not show any obvious trend. The standard deviations in some treatment groups are particularly high as occasionally one fish in the group had a much higher tail moment than the other individuals. These different "outlier" values are however within value ranges that can be observed in case of DNA damage and even though cannot be solely attributed to the treatment experienced they have not been excluded from the analysis. In addition, for each treatment, 10 samples were collected for Comet assay and duplicate wells were placed on the slide, however occasionally one or both the wells for an individual were lost during the electrophoresis step of the assay reducing the sample size for analysis. There was no effect of exposure to copper and chromium at either of the concentrations tested and exposure route used. Exposure to lead had an effect at low concentrations if presented via the food and at high concentration when presented via the sediment. Exposure to zinc had an effect at both concentrations when zinc was present in both the sediment and the food pellet. The metal mixture had a

negative effect only at low concentration via the sediments (Figure 4.4). Those results are somewhat difficult to interpret, but it is worth noting that increase in DNA damage in control fish has been recorded in a study by Nogueira et al. (2009) where it was also observed that in long term exposure of glass eels to 7,12-Dimethylbenz[a]anthracene (DMBA) only higher concentrations of contaminants produced long lasting DNA damage in blood cells, while 1-3 days after exposure the DNA damage observed was very high for all exposure concentration tested and low in control fish. Similarly, eels exposed to environmentally relevant concentrations of the pesticide Roundup showed an increase in total DNA damage after one or three-day exposure, but when the damage was divided into classes there was a time-related difference in the magnitude of damage with higher classes of damage prevalent early on in the exposure period (Guilherme et al. 2010). Similar results were obtained by Saleha Banu et al., (2001) when they investigated the response of tilapia (*Tilapia mossambica*) to various concentration of a pesticide over time. This study found that there was a dose-related increase and a time dependent decrease in the DNA damage measured returning to control level by 96 hr.

In this study, eels were exposed to metals for a period of 5 weeks, and there was no clear effect on the DNA damage measured by the Comet assay. However, in the study with glass eels described in chapter 3, where fish were exposed only for 2 weeks there was a statistically significant effect of the metal exposure (at least in all the lower concentrations) and the amount of DNA damage. The different results between those two studies could be due to the different life stages investigated, with the first study carried out on glass eels and the one presented in this chapter on older pigmented elvers. Another difference is in the route of exposure which was via water with glass eels but via sediment and/or food with the elvers. This was to better represent the typical environment and lifestyle of these 2 eel stages. Alternatively, the lack of significant DNA damage in the eels exposed for 5 weeks could be due to DNA repair processes that take place shortly after exposure, and therefore compensate for damage that may have occurred earlier on during exposure.

PCR results

The liver taken from each eel was used to extract RNA as described in Section 2.3.4 in order to quantify the corresponding level of gene expression of two genes linked with detoxification, cytochrome P4501A (CYP) and metallothionein (MT) and two housekeeping genes, elongating Factor-1 (EF1) and Ribosomal protein L13a (L13). The efficiency of the PCR for each gene was 1.87 (CYP), 1.80 (MT), 1.86 (EF1) and 1.84 (L13).

CYP genes code for a large family of enzymes involved in oxidative metabolism and biotransformation of toxicants such as chlorinated and aromatic hydrocarbons (Maes et al., 2013). MT is implicated in detoxification of metals (Gorbi et al., 2005) and has an antioxidant function involved in the homeostasis of essential metals like zinc and copper (Maes et al., 2013).

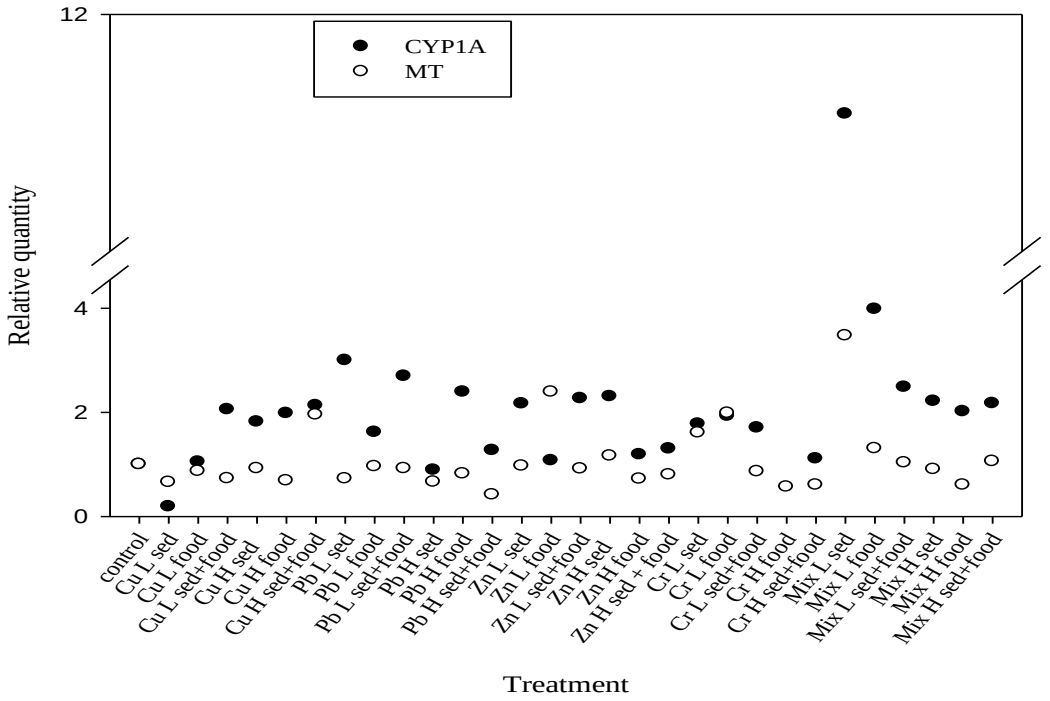


Figure 4.5 Results of gene expression measurement. Relative quantity of each gene expression versus its control as calculated by REST.

There was no significant difference in gene expression between any of the various treatments (Figure 4.5). This may imply that exposure to the metals and concentration tested had no negative effect on the eels. However, caution should be taken in drawing

such firm conclusions from the PCR results observed in this experiment. Some studies on eels have found clear dose-response pattern between gene expression (of detoxifying genes) and pollutant concentration (Aubry et al., 2007, 2008 for CYP1A and Pierron et al., 2007 for MT), while another study found high level of variation in gene expression in eels originating from scarcely polluted areas whereas eels from more polluted areas showed a down-regulated level of gene transcription with increase in pollution level (Maes et al., 2013). Bird et al. (2008) indicated that concentrations of MTs in the liver of eels are closely correlated to the concentrations of metals in the liver, but this does not strictly represent the metal concentration observed at locations where the eels were caught. Both CYP1A (Gorbi & Regoli, 2004) and MTs (Bourdineaud et al., 2006) genes have also been shown to be differently expressed during different seasons. In particular, Maes et al. (2013) suggested that gene transcription levels increase linearly only at low pollution levels of single toxicants and in healthy organisms, whereas at high pollution level gene transcription is inhibited or even stopped. This could be a protective mechanism of chronically exposed individuals to avoid over-expression of detoxification genes which have deleterious effects at high expression levels (Barouki & Morel, 2001; Marohn et al., 2008; Kessabi et al., 2010). A study on juvenile eel exposed to 3.9M DMBA over a month indicated that CYP1A expression was elevated in eels sampled after seven-day exposure but the expression was similar in control and exposed eels after 28 days (Nogueira et al., 2009) suggesting that long term or chronic exposure may not elicit the same increased gene expression observed in short term exposure. Additionally, the low sample size for some parameters and the absence of treatment duplication could have failed to show subtle differences that could have otherwise been quantified.

4.4. Eel exposure and olfaction

4.4.1. Introduction

In teleost fish, the sense of smell (olfaction) is one of the principal sensory systems controlling and mediating a wide range of physiological processes and behaviours (see Hara, 1994; Kerman et al., 2013 for reviews). Olfaction has been shown to be involved in migration and homing (Scholz et al., 1976; Hasler & Scholz, 1983; Nevitt et al., 1994; Dittman & Quinn, 1996; Dixson, et al., 2008; Vrieze et al., 2010; Bandoh et al., 2011;), reproduction (Moore & Waring, 1996; Stacey & Sorensen, 2008; Belanger et al., 2010; Sorensen & Hove, 2010; Johnson et al., 2012), feeding (Hamdani et al., 2001; Kerman et al., 2013 for review), predator and prey detection (Hara, 1993; Johannesen et al., 2012, 2014), social interactions (Gilmour et al., 2005), shoaling (Ward et al., 2002;) and kin recognition (Moore et al., 1994; Olsen & Winberg, 1996; Olsen, 1992; Hesse et al., 2012). Fish are known to detect a wide diversity of dissolved chemicals through the olfactory system and which range from specific chemicals derived from the geology and vegetation within a river catchment to specific pheromones that mediate species specific reproduction.

The European eel has a well-developed sense of smell (Tesch, 2003). Olfaction is known to be important throughout the life history of the fish mediating migration, feeding and potentially reproduction. The returning juvenile glass eels are considered to use a range of olfactory cues to orientate within coastal waters and estuaries.

Odorants derived from the vegetation and soil have been identified as causing chemoreception in eels (Tosi & Sola, 1993; Sola, 1995), whilst Sorensen (1986) considered that micro-organisms responsible for detrital decomposition could be the source of this response in the American eel (*Anguilla rostrata*). Sola & Tongiorgi (1996) studied the behaviour of upstream migrating glass eels to salt and brackish water solutions of eight pure organic earthy and green odorants. The strong attraction that glass eels showed to earthy and green odorants emerged as the level of salinity was reduced, suggesting that these chemicals could be orienting cues in the last phase of glass eel migration as they move from coastal waters and into freshwater. The role of con-specific odour is also considered to be an attractant (Miles, 1968; Pesaro et al., 1981; Sorensen, 1986) and pheromones were suggested to have a role in eel migration

(Miles, 1968) although Sorensen (1986) suggested that they were only involved in close-range attraction and gathering behaviour, because the dilution of odour was too large to allow a detection within the coastal zone. Other odorants studied have included eel skin mucus (Saglio, 1982), bile salts (Sola & Tosi, 1993) as well as several amino acids (Crnjar et al., 1992; Sola & Tongiorgi, 1998). Crnjar and colleagues (1992), carried out electro-olfactograms (EOGs) from both unpigmented and pigmented elvers and showed that various amino acids (glycine, L-alanine, L-valine, L-leucine, L-asparagine, L-glutamine and L-methionine) were detected by the olfactory epithelium of the fish. Barbin (1998) investigated the role of olfaction in homing migrations of American eels (*Anguilla rostrata*) in an estuary using acoustic telemetry and yellow eels displaced from a capture site. The author considered that olfaction was important for discrimination of the appropriate tide for upstream transport and location of a home site but was not the only orientational mechanism used in estuaries. Mechanisms used to detect rates of change of water mass characteristics were probably important for guidance during estuarine migrations. Tosi et al. (1990) also considered that salinity and temperature were more important than odour in determining glass eel orientation in coastal waters and estuaries.

Olfaction is also important in adult eels. Huertas et al. (2007; 2010) have shown the olfactory potency of conspecific bile fluid and skin mucus in the European eel *Anguilla anguilla*. The authors suggest that bile acids may have potential roles in both intra-specific chemical communication and in inter-specific interactions and that the results of their studies are consistent with a role for chemical communication in the reproduction of the European eel. However, the olfactory organs and retinal structure were examined in sexually immature and artificially matured female *Anguilla anguilla* (Pankhurst & Lythgoe, 1983) and were found to be atrophied in artificially matured eels of advanced development. The density of mucous cells in olfactory lamellae decreased from a maximum of 441 mm^{-2} in sexually immature eels to as low as 19 mm^{-2} in sexually maturing eels. The authors suggested the changes in vision and olfaction indicate a change in the relative importance of the two sensory modalities with sexual maturation. Unfortunately, the absence of any research on the reproductive behaviour of European eels in the wild does not allow a true assessment of the role of olfaction in eel reproduction, although studies on the many other species of fish would suggest that the sense of smell plays a key role (Burnard et al., 2008).

Olfaction is also considered to play a major role in the feeding behaviour of the European eel. The majority of eels are considered to feed at night on benthic organisms when light levels are low and vision may play a reduced role in prey detection and feeding. During this period, olfaction may be the prevalent sense eel use. Walker et al. (2014) indicated that activity in resident yellow eels within the tidal waters of the rivers Frome and Piddle was generally, but not exclusively, nocturnal, with the start and end times closely associated with sunset and sunrise, respectively. This behaviour was probably related to foraging and feeding. Atta, (2013) showed that the surface area of the olfactory lamellae in the eel was found to be about 590.9% of the retinal area. Thus, the olfactory organs are well developed in the eel and the author also concluded that the olfaction plays an important role in their feeding habits.

The sensory cues involved in the seaward migration of silver eels are not known although magnetic orientation is considered to be important (Moore & Riley, 2009; Durif et al., 2013). Olfaction is not known to be involved in the oceanic spawning migration although Westin (1990) considered that the sense of smell may be important in allowing the eels to successfully migrate from the Baltic Sea.

It is possible that contaminants may also affect the sense of smell in the eel and if this is the case there may be a deleterious effect on feeding, growth, fat deposition, metabolic activity and subsequently successful spawning migration. Contaminants can act as signals, modify odorant perception and/or interfere with the nervous system and/or other physiological responses not directly through olfaction which could result in alteration of normal olfactory-mediated responses (Tierney et al 2010).

4.4.2. Fenitrothion and Electro-olfactogram

4.4.2.1. Methods

Laboratory studies were carried out to assess the effect of fenitrothion on eel olfaction and in particular the ability to detect amino acids and bile acids. Fenitrothion was selected as it is an organophosphate (OP) insecticide similar to diazinon which is commonly used as the active ingredient in dips to control parasites on sheep and has previously been shown to inhibit the sense of smell in salmon (Moore & Waring, 1995).

Fenitrothion (O,O-dimethyl O-4-nitro-m-tolyl phosphorothioate, CAS Nr 122-14-5; Figure 5.6) is a contact organophosphate insecticide that has been widely used since 1959 to control insects in agriculture and for fly, mosquito and cockroach control in public health programmes worldwide.

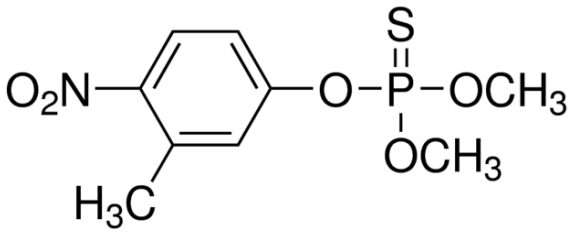


Figure 4.6 Fenitrothion chemical structutre.

Since 2001, fenitrothion has not been approved for use in the UK as an agricultural plant protection product although it was still permitted to be used as an insecticide for non-agricultural purposes (Advisory Committee on Pesticides, 2006). In 2007, the Commission of the European Communities agreed on the withdrawal of fenitrothion for use in plant protection products to be implemented later the next year (2007/379/EC), and from this date no authorizations for plant protection products containing fenitrothion can be granted or renewed.

Fenitrothion is not persistent in soil and leaching from agricultural land into water courses is considered not to be significant (WHO, 2010). Therefore, there is negligible risk of contamination to groundwater as a result of leaching. However, aquatic organisms (fish and invertebrates) are potentially at risk, especially in the event of overspray from non-agricultural purposes to static and/or running water bodies.

Fenitrothion dissipates quite quickly in microbially active natural water systems and it has a half-life of less than one week. Fenitrothion as a compound tends to migrate to the sediment in many water courses (WHO, 2010) and it has been measured in a number of water courses in England. Typically, levels are less than 0.001 μg l^{-1} but periodically spikes occur with levels as high as 0.1 μg l^{-1} being recorded in the River Avon, Hampshire (Environment Agency Pesticide Monitoring Data 1997-2009).

Fenitrothion is known to be toxic to a number of fish. The 96-hour LC_{50} for brook trout was shown to be 1.7 mg l^{-1} and 3.8 mg l^{-1} for bluegill sunfish (US Environmental Protection Agency, 1987). Takimoto et al. (1987) estimated a 96 hour LC_{50} of 3.5 mg l^{-1} for adult killifish *Oryzias latipes* and 2.6 mg l^{-1} for the mullet *Mugil cephalus*. The LC_{50}s for both fish in freshwater were also similar, at 3.5 and 2.6 mg l^{-1} respectively. In the European eel the 96-hour LC_{50} is 0.2 mg l^{-1} (Ferrando et al., 1991).

In November 2009, adult eels were obtained from the River Stour (Hampshire) and maintained at the Cefas Lowestoft Laboratory as described in Section 2.1. The eels were exposed in flow through freshwater to an environmental concentration of fenitrothion (0.05 μg l^{-1}) or a control (no fenitrothion) for three weeks. Exposure was achieved and maintained using a peristaltic pump as described in Section 2.4. At the end of the 3 weeks, the olfactory responses of each eel to three odorants were measured using an electrophysiological technique (electro-olfactogram: EOG) (Moore & Waring, 1995, 1998; Waring & Moore, 1997) described in Section 2.6. EOG recording measures trans-epithelial voltage gradients from the surface of the olfactory epithelium (olfactory receptors) and is considered to reflect multi-unit cell activity. The three odorants tested were water solutions of glutamine (10^{-3}M), bile from gall bladders of eels (1:200 dilution) and ecdysone (10^{-5}M). Each of the odorant was delivered to the olfactory epithelium using a multichannel, computer-controlled perfusion system. At the end of each recording the eels were sacrificed and morphological and physiological parameters were measured.

4.4.2.2. Results and discussion

The physiological and morphological measurements taken from all fish after completion of the EOG recordings are shown in Table 4.7. When fish were sampled a record was made on the level of the parasite *Anguillicola* infestation in the swim bladder of each fish. The records for all fish (both control and exposed) indicate that 23% of the eels had no parasite, 29 % had few parasite (between 1 and 5), 6% had some (between 6 and 10) and 42% had many (more than 10). After exposure to a 0.05 $\mu g\ l^{-1}$ concentration of fenitrothion, the electrophysiological responses recorded from the olfactory epithelium of the eels to all three odorants were similar to the responses recorded from the control fish (Table 4.8). The amplitude of each EOG response was measured from the baseline of the peak of each phasic displacement and expressed in mV (see Figure 4.7 for the visual output of the phasic displacement).

The results suggest that the pesticide has no effect on the olfactory ability of the eel. However, all the eels showed a response to the compound ecdysone. Ecdysone is a steroidal pro-hormone of the major insect moulting hormone 20-hydroxyecdysone. It is also produced by crustacean during moulting and growth. This is the very first evidence that a fish is able to detect via the sense of smell this hormone and may be the major mechanism as to how eels detect prey during the freshwater stage of their life cycle.

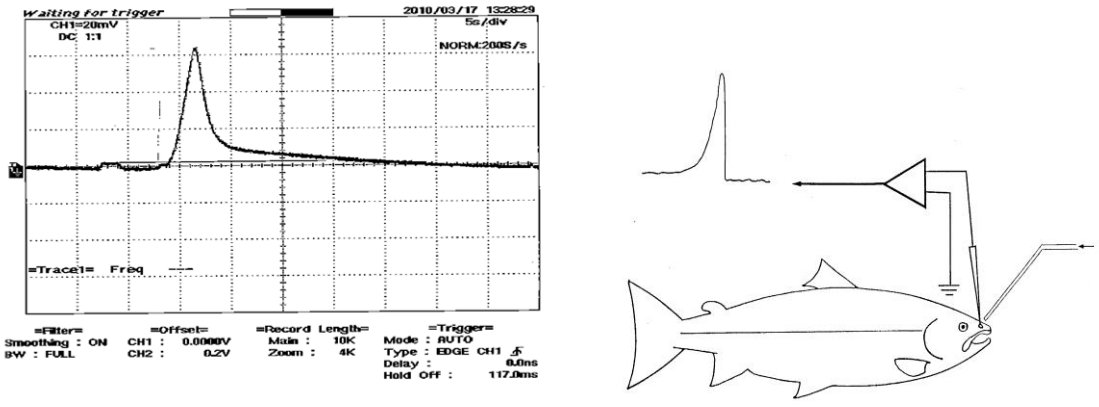


Figure 4.7 Output of EOG response to glutamine. Diagram of electrode position, stimulus and output of response.

Table 4.7 Physiological measurements of silver eels exposed to 0.05 µg l^{-1} of fenitrothion for 3 weeks.

Parameters	**Control**			**Exposed**			**t-test**
	Mean	sem	N	Mean	sem	N	P value
weight (g)	379.8	26.5	16	380.3	26.7	15	0.991
length (cm)	59.26	1.215	16	59.560	1.337	15	0.868
condition factor	0.179	0.005	16	0.176	0.004	15	0.724
eye index	7.700	0.315	16	7.596	0.223	15	0.789
fat content (%)	19.225	0.626	16	19.000	0.550	15	0.789
hepatosomatic index	1.374	0.065	15	1.260	0.050	14	0.181
gonadosomatic index	1.867	0.064	16	1.878	0.063	15	0.906
gill Na/K ATPase (µmol Pi (mg hr)$^{-1}$)	11.362	1.381	16	9.845	1.169	15	0.409
haematocrit (%)	28.139	2.510	9	28.615	1.082	13	0.865
osmolarity (mosm kg water^{-1})	292.286	8.289	14	294.467	8.750	15	0.858
Cl^- (mM)	83.786	3.337	14	79.200	3.626	15	0.360
Na^+ (mM)	168.250	4.083	14	163.539	2.783	15	0.350
K^+ (mM)	3.197	0.195	14	3.701	0.303	15	0.175
glucose (mmol l^{-1})	2.651	0.260	14	2.193	0.222	12	0.193

Table 4.8 EOG response of silver eels exposed to 0.05 µg l^{-1} of fenitrothion for 3 weeks.

Parameters	**Control**			**Exposed**			**t-test**
	Mean	sem	N	Mean	sem	N	P value
EOG - 10^{-3}M glutamine (mV)	1.714	0.300	14	1.964	0.349	13	0.592
EOG - 10^{-1}M glutamine (mV)	4.873	0.513	15	4.938	0.755	15	0.944
EOG - bile (mV)	2.124	0.196	15	2.244	0.338	14	0.762
EOG - 10^{-5}M ecdysone (mV)	2.169	0.399	7	3.546	0.864	7	0.184

4.4.3. Atrazine and Electro-olfactogram

Similar laboratory studies were carried out to assess the effect of the pesticide atrazine on olfactory function in eels.

In 2004 a total ban on the use of atrazine was implemented by the EU. However, atrazine was included in the present study as it is a good example of a triazine pesticide and previous studies have indicated that it inhibits the sense of smell in salmon (Moore & Waring 1998).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine, CAS Nr 1912-24-9; Figure 4.8) is a water-soluble pre- and post-emergence herbicide for the control of annual and perennial grass and annual broad-leaved weeds and it has been one of the most widely used herbicides in past decades.

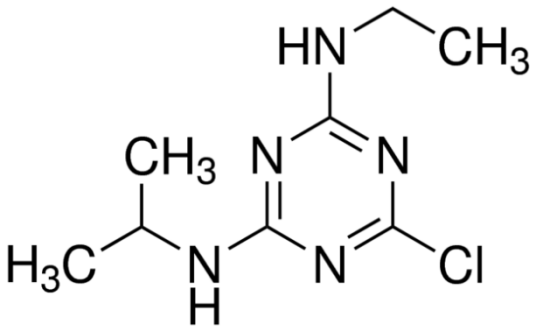


Figure 4.8 Atrazine chemical structure.

Atrazine is known to have high mobility through soil and is a known contaminant of aquatic ecosystems in England and Wales (Rivera et al., 1986; Solomon et al., 1996; Power, 1999) where it has been detected in surface waters at concentrations exceeding 0.1 µg l^{-1} in some areas of England (Environment Agency, 1997). In 1992 and 1993, atrazine was one of the 5 pesticides most frequently present in both ground and surface water at levels in excess of the Maximum Admissible Concentration 0.1 µg l^{-1} imposed by the Water Act 1991. In addition, analyses of UK surface waters demonstrated levels exceeding the proposed Environmental Quality Standard of 2.0 µg l^{-1} based on the annual combined average of atrazine and simazine. Concentrations up to 275 µg l^{-1}

have been detected in run-off water from agricultural land, but at these concentrations it is considered not to be a risk to aquatic life. Since 1993, the use of atrazine has been banned on non-cropped land. The use of atrazine in the UK was banned in 2007 and as a result there has been a decline in its detection in UK surface waters. However, it is still measured during routine monitoring by the Environment Agency in a number of catchments. Atrazine is still licensed for use in North America although in 2012 the FIFRA Scientific Advisory Panel recommended that the US Environmental Protection Agency further analyse existing data and proposed that additional studies be conducted to further refine the environmental fate and ecological risk assessment for atrazine. The Panel also recommended some refinements and alternative approaches to consider when interpreting uncertainty in atrazine water monitoring data. The atrazine registration review began in 2013. Although primarily aimed at reversibly inhibiting photosynthesis (Tietjen et al., 1991), atrazine has also been found to affect a variety of physiological processes in aquatic animals. Animals have been found to accumulate it in a variety of tissues (Gunkel & Streit, 1980; du Preez & van Vuren, 1992). In fresh water invertebrates, atrazine has been found to affect hydromineral balance or gill function in crabs (Prasad et al., 1990; Silvestre et al., 2002) and hemocyanin function (Prasad et al., 1995). In fish, atrazine has been shown to affect haematology by altering the constituents of the blood (Prasad et al., 1991; Hussein et al., 1996) and metabolism, with changes in oxygen consumption and the lipid profile in liver and muscle (Grobler et al., 1989; Srinivas et al., 1991; Prasad et al., 1995). Saglio & Trijasse (1998) showed that a 24-hour exposure to low levels of atrazine (0.5-5 $\mu g\ l^{-1}$) significantly affected the behaviour of goldfish which displayed a burst swimming reaction when exposed to atrazine, a decrease in their grouping behaviour and an increase in surfacing activity. In Atlantic salmon, atrazine has been shown to affect pheromonal detection by mature male parr (Moore & Waring, 1998; Moore & Lower, 2001). More recently low, ecologically relevant levels of atrazine have been shown to impair sexual development in male frogs (Hayes et al., 2002). For a recent review on the effects of atrazine see Van der Kraak et al., (2014).

4.4.3.1. Methods

In summer 2011, 10 non-migratory yellow eels were collected from the River Avon (Hampshire) and brought to the Cefas Lowestoft Laboratory. Here fish were equally divided over two identical 700 l tanks with flow through ambient freshwater (temperature 17.3-19.2°C) and naturally simulated photoperiod. Fish were fed daily with defrosted sandeel (*Ammodytes ssp*). After a two-week acclimation period, a peristaltic pump was set up by the tanks as described previously. One tank served as control and the other was exposed to atrazine at a concentration of 1 µg l^{-1} for a period of two weeks. During these two weeks, food consumption in each tank was monitored daily to assess potential effects of exposure to feeding behaviour. At the end of the exposure period each fish was assessed for the olfactory response (EOG) to a known odorant, glutamine at a 10^{-1}M concentration. At the end of each experiment the eels were sacrificed and morphological and physiological measurements were obtained.

4.4.3.2. Results and discussion

Data on the level of swim bladder infestation by the parasite *Anguillicola* indicates that 60% of the eels sampled (both control and exposed) had no parasite while 40% had some level of infestation.

Exposure to the pesticide atrazine had no effect on food consumption and on the olfactory response to glutamine or any of the measured physiological and morphological parameters (Table 4.9) with the exception of a small decrease in kidney ATPase in exposed eels. The difference in the level of ATPase observed in exposed fish could be due to a disturbance in the ion exchange activity in the kidney similary to what was suggested by Waring & Moore (2004) for their observed decrease in gill ATPase in salmon smolts exposed to atrazine.

Table 4.9 Morphological and physiological measurements and EOG responses from adult eels exposed to 1 µg l^{-1} of atrazine for two weeks.

Parameter	**control** (N=5)		**exposed** (N=5)		t-test
	mean	sem	mean	sem	P value
weight (g)	405.9	62.2	338.3	51.1	0.493
length (cm)	62.54	3.066	60.220	2.963	0.603
condition factor	0.16	0.009	0.151	0.009	0.560
eye index	7.23	1.034	6.498	0.567	0.611
fat content (%)	24.90	1.366	22.180	4.368	0.563
hepatosomatic index	1.07	0.068	1.350	0.241	0.342
gonadosomatic index	1.08	0.205	1.247	0.074	0.125
gill Na/K ATPase (µmol Pi (mg hr)$^{-1}$)	7.10	0.807	8.638	1.871	0.535
kidney Na/K ATPase (µmol Pi (mg hr)$^{-1}$)	15.37	1.506	9.532	2.171	0.048
EOG to 10^{-1}M glutamine (mV)	6.66	1.126	5.538	2.446	0.700

The result of the Electro-olfactogram differs from previous work on Atlantic salmon smolts, where short term exposure of the olfactory epithelium of male parr to concentrations of the pesticide ranging from 2.0 - 20 µg l^{-1} significantly reduced the olfactory response to prostaglandin $F_{2\alpha}$, which is considered to be a reproductive priming pheromone in this species (Moore & Waring, 1998). In addition, exposure of male parr to atrazine significantly reduced their ability to respond to the reproductive priming effect of ovulated female salmon urine. The reproductive priming effect on milt and plasma 17,20β-dihydroxy-4-pregnen-3-one (the pre-ovulatory sex steroid) levels were reduced at water atrazine concentrations at and above 0.04 µg l^{-1}. This is considered to be due to the direct impact of atrazine on olfactory mediated reproduction (Moore & Waring, 1998). Why there is such a difference in response to

atrazine between the eel and the salmon is not known. Both species have very similar olfactory systems and detect similar groups of odorants (see Tesch, 2003). However, the eel is known to reside for longer periods in freshwater than salmon and is exposed to a wide suite of contaminants. The previous exposure of the experimental eels to the pesticide atrazine is not known. Although the pesticide was present in the river from which the eels were sourced exposure rates and durations are not understood. Therefore, one explanation for the difference in response is that the experimental eels may have already been exposed to the pesticide and have in some way adapted or acclimatised to its physiological and neurological effects. Previous studies have indicated that the olfactory system of fish is able to acclimatise to the exposure of water borne contaminants such as copper (Saucier& Astic, 1995). It would have been informative to measure the atrazine loading of the eel tissue and bioaccumulation of the pesticide to determine whether the eel had been exposed to significant levels of the contaminant prior to this study.

Chapter 5. Migratory stage

5.1. Introduction - transition from fresh to saltwater

Eels undergo a physiological and morphological transformation during which they develop from the freshwater yellow eel to the migratory form, a process that is known as 'silvering' and which enables them to successfully perform their transoceanic spawning migration. During this process there is an increase in the size of the eye, changes in fin length, an increase in maturation parameters such as Gonadosomatic Index (GSI), changes in the Hepatosomatic Index (HSI), and increases in the fat content. The silvering process also includes physiological changes (e.g. salinity tolerance, increased hypo-osmoregulatory ability), which allow the eel to better adapt and survive within the marine environment. The objective of the work presented in this chapter was to examine how contaminants could potentially modify and regulate key physiological and morphological parameters associated with silvering process and so effect the ability of migrating eels to survive once they had entered the sea.

The contaminants investigated were the ones identified from the literature (Heberer, 2002; Buchanan et al., 2009; Garthwaite et al., 2010; Stuart et al., 2011) and monitoring data (Environment Agency) that would be both temporally and spatially relevant to the silvering process. Specifically, the research examined the effect of contaminants on plasma ion concentrations, gill and kidney Na/K ATPase activity and survival to saltwater challenge tests. In the saltwater challenge test fish that were held in freshwater are transferred in seawater for a period of 3-5 days to assess whether the fish had successfully completed all the physiological adaptation required prior to the transition from fresh to saltwater. In addition, other measures such as metabolic capability were also considered.

5.2. Silver eel and fenitrothion

5.2.1. Methods

Fenitrothion is an insecticide and has been described in detail in Section 4.4.2.1.

Although fenitrothion is fat-soluble, the rates of biotransformation and the excretion of metabolites largely mitigate bioconcentration in animals, although the high fat content of European eels may make them more susceptible than other freshwater fish species. Typically, the duration of exposure is brief in the aquatic environment as fenitrothion dissipates quite quickly in microbially active natural water systems but it still has a half-life of less than one week. Because of its tendency to migrate to the sediment in many water courses (WHO, 2010) it may pose an additional problem to eels, principally during the growing stage where they are buried within the sediment during daylight hours.

Fenitrothion is reported to be a neurotoxin in fish due to its irreversible inhibitory effect on acetylcholinesterase – AChE (Sancho et al., 1997b). The enzyme AChE degrades the neurotransmitter acetylcholine in cholinergic synapses. The inhibition provokes an accumulation of acetylcholine in synapses with disruption of the nerve function that can lead to a wide range of toxic effects in fish (Kapur et al., 1978; Morgan et al., 1990; Sebire et al. 2009). Fenitrothion is also known to have a wide range of sub-lethal effects on the physiology of the European eel. These include effects on the energy metabolism where there is an observed increase in blood glucose and lactate levels, increase in liver and gill lactate, while blood proteins decrease during exposure (Sancho et al., 1997a; 1998), inhibition of brain AChE (Sancho et al., 1997b) and alteration of muscle physiology (Sancho et al., 1999) where both protein and lipid levels in eel muscles decreased after exposure. Research has also indicated that fenitrothion may inhibit gill Na^+/K^+ATPase activity (Sancho et al., 1997c). These studies however have investigated high concentrations of fenitrothion (1 to 200 µg l^{-1}) which are unlikely to be found in the environment unless a spill has occurred.

During the transition from freshwater to the marine environment there are significant changes to the gill ATPase activity as well as plasma ion concentrations that permit the fish to successfully osmoregulate in its new environment. Therefore, a laboratory based experiment was undertaken to determine whether environmental levels of

fenitrothion had an impact on a range of physiological and morphological parameters associated with the seawater adaptation of wild silver eels and their ability to survive in full strength sea water. The experiment was designed to examin three different environmental concentrations of the pesticide: Low 0.001 μg l^{-1}; Medium 0.01 μg l^{-1}; High 0.05 μg l^{-1}.

In November 2009, 59 silver eels were obtained from a commercial eel fishermen operating on a trap on the River Stour (Hampshire) during the eel spawning migration. After collection, eel were transported to the Cefas Lowestoft Laboratory as described in Section 2.1. In the laboratory, 54 of the collected fish were acclimatized for seven weeks in eight identical indoor 550 l freshwater tanks as described in Section 2.1. The water temperature during both acclimation and the experiment ranged from 4-11 °C. Fish were fed daily with de-frosted sand eel *Ammodytes spp.* (Monday to Friday) during acclimation. Most food was not consumed as eels are thought to stop feeding when they metamorphose to the silver stage (van Ginneken et al., 2007a).

In order to determine the general health of the population of eels from the River Stour, used in this experiment, the remaining 5 fish were screened by the Fish Health Inspectors at Cefas Weymouth Laboratory. This was considered necessary within the context of the present research in order to establish whether any effects shown by exposure to fenitrothion may have been complicated by the general health of the eels. The screening involved a visual examination of the fish, inspection for known eel parasites and determination of the presence of VHS (viral haemorrhagic septicaemia), IHN (infectious haematopoietic necrosis), IPN (infectious pancreatic necrosis), SVC (spring viraemia of carp) and eel rhabdovirus.

At the end of the acclimation period, the eels were exposed in freshwater to the 3 different concentrations of fenitrothion for a period of two weeks. Exposure was carried out in flow-through conditions maintaining the desired concentration of fenitrothion in the tanks using a peristaltic pump as described in Section 2.4 (Figure 5.1).

Figure 5.1 Tank and peristaltic pump set up for silver eel experiment.

Each of the three concentrations was tested in duplicate tanks and 2 additional tanks were used as controls. At the end of the two-week exposure, half of the fish were sacrificed and sampled for the physiological biomarkers associated with saltwater adaptation, and the remaining fish were transferred to saltwater for 72 hours (saltwater challenge test) to monitor survival. Fish surviving the saltwater challenge test were then sampled for the same physiological biomarkers associated with saltwater adaptation. The data were analysed using a 2-way ANOVA. To isolate which groups differed from one another this was followed by a Multiple Comparison Procedure (Holm-Sidak Method). The significance level was set at 0.05 and the P value is reported.

5.2.2. Results and discussion

Assessment of the number of eel parasite *Anguillicola* in the swim bladder of all the eels sampled indicated that 13% did not have any parasite, and the remaining 87% had each more than 5 parasites. Exposure of eels to fenitrothion in freshwater for two weeks had no effect on any of the measured morphological or physiological biomarkers when compared to the control (Table 5.1). Similarly, after the fish had been transferred to saltwater for 72 hours, no mortalities occurred and the eels showed no significant effects of exposure to any of the three concentrations of fenitrothion when compared to the control (Table 5.2).

Table 5.1 Morphological and physiological data from silver European eels exposed to low, medium or high concentrations of fenitrothion in freshwater for 2 weeks and then sampled prior to a 72-hour saltwater challenge test.

Freshwater	**Control (N=7)**		**low (0.001 µg l^{-1}) (N=6)**		**medium (0.01 µg l^{-1}) (N=7)**		**high (0.05 µg l^{-1}) (N=6)**		**ANOVA**
	mean	*sem*	*Mean*	*sem*	*mean*	*sem*	*mean*	*sem*	*P value*
length (cm)	58.61	1.8	58.35	2.25	58.47	2.37	59.08	2.27	0.996
weight (g)	411.4	55.02	374.6	43.7	401.83	30.76	397.9	41.29	0.945
condition factor	0.198	0.013	0.184	0.006	0.206	0.021	0.189	0.006	0.275
eye index	9.91	0.53	9.67	0.8	9.16	0.85	9.52	0.41	0.877
fat content (%)	18.26	0.38	19.63	0.65	20.67	0.6	19.45	0.75	0.054
hepatosomatic index	1.601	0.086	1.405	0.093	1.588	0.125	1.484	0.037	0.437
gonadosomatic index	1.678	0.106	1.772	0.121	1.662	0.098	1.853	0.092	0.555
gill Na/K ATPase (µmol Pi (mg hr)$^{-1}$)	14.88	2.49	17.51	2.95	12.91	1.84	15.5	1.35	0.554
osmolarity (mosm kg water^{-1})	333	8.649	368.3	10.2	349.6	8.766	356	6.455	0.058
Cl- (mmol l^{-1})	111.7	5.24	114.67	5.75	118.3	4.602	116.8	4.269	0.793
Na+ (mM)	181.7	5.29	182.27	4.204	189.4	6.262	184.5	3.926	0.696
K+ (mM)	4.234	0.501	4.318	0.496	3.702	0.333	3.511	0.331	0.482
glucose (mmol l^{-1})	2.624	0.361	3.599	0.267	2.948	0.345	3.479	0.307	0.154

Table 5.2 Morphological and physiological data from silver European eels exposed to low, medium or high concentrations of fenitrothion in freshwater for 2 weeks and then sampled after a 72-hour saltwater challenge test.

Saltwater	control (N=7)		low (0.001 µg l^{-1}) (N=7)		medium (0.01 µg l^{-1}) (N=7)		high (0.05 µg l^{-1}) (N=6)		ANOVA
	mean	*sem*	*mean*	*sem*	*mean*	*sem*	*mean*	*sem*	*P value*
length (cm)	57.84	4.43	55.6	1.79	56.45	1.9	56.28	2.56	0.954
weight (g)	389.04	75.8	324.4	31.25	326.49	31.2	345.4	46.42	0.773
condition factor	0.181	0.005	0.186	0.007	0.179	0.006	0.188	0.006	0.696
eye index	8.8	0.19	9.59	0.34	9.78	0.42	9.89	0.32	0.104
fat content (%)	18.16	0.94	19.44	0.75	19.2	0.75	18.8	0.8	0.687
hepatosomatic index	1.489	0.091	1.476	0.086	1.607	0.099	1.359	0.12	0.405
gonadosomatic index	1.668	0.063	1.569	0.065	1.615	0.102	1.603	0.044	0.826
gill Na/K ATPase (µmol Pi (mg hr)$^{-1}$)	16.81	2.53	13.45	1.63	14.76	2.16	9.61	1.14	0.094
osmolarity (mosm kg water^{-1})	382.1	13.14	389.9	7.766	400.1	10.42	404.8	14.2	0.518
Cl- (mmol l^{-1})	139.4	3.199	139.4	3.316	140	4.271	147.7	5.719	0.465
Na+ (mM)	191.3	3.066	194.7	2.132	193.7	3.661	196.3	4.889	0.789
K+ (mM)	3.461	0.161	3.528	0.203	3.355	0.297	3.343	0.43	0.25
glucose (mmol l^{-1})	2.598	0.628	2.109	0.147	2.336	0.312	2.4	0.255	0.901

The transfer of the eels from freshwater to saltwater did not have a significant effect on the levels of gill Na^+/K^+ATPase activity in either the controls or any of the 3 treated groups (Figure 5.2). There was a similar trend in terms of plasma Na^+ and K^+ ions. These results differ from similar studies on the Atlantic salmon where there is an increase in gill ATPase activity and plasma sodium and potassium ions (Waring & Moore, 2004).

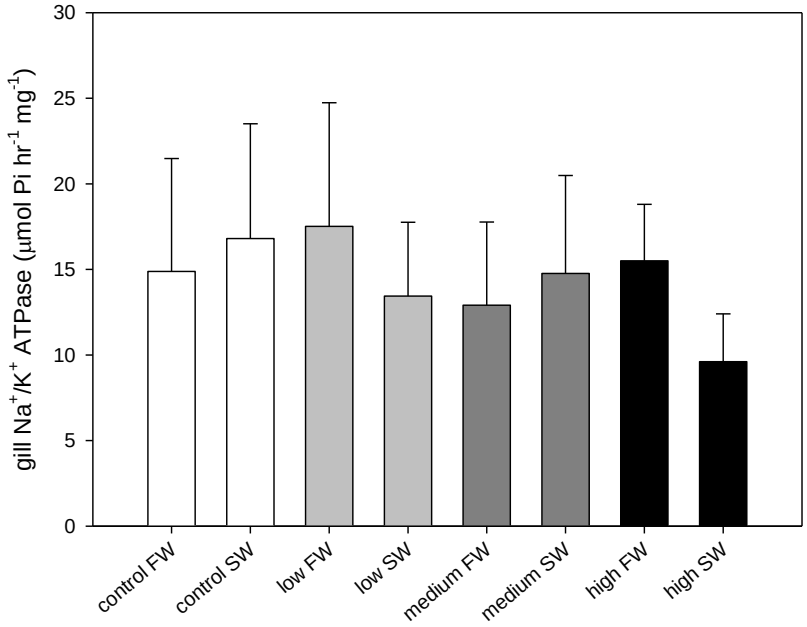


Figure 5.2 Gill Na+/K+ATPase activity in eels exposed to concentrations of 0.001 µg l-1(Low), 0.01 µg l-1 (Medium) and 0.05 µg l-1 (High) fenitrothion in freshwater (FW) and after the saltwater challenge (SW). The data represents mean ± SD of 7 eels per group.

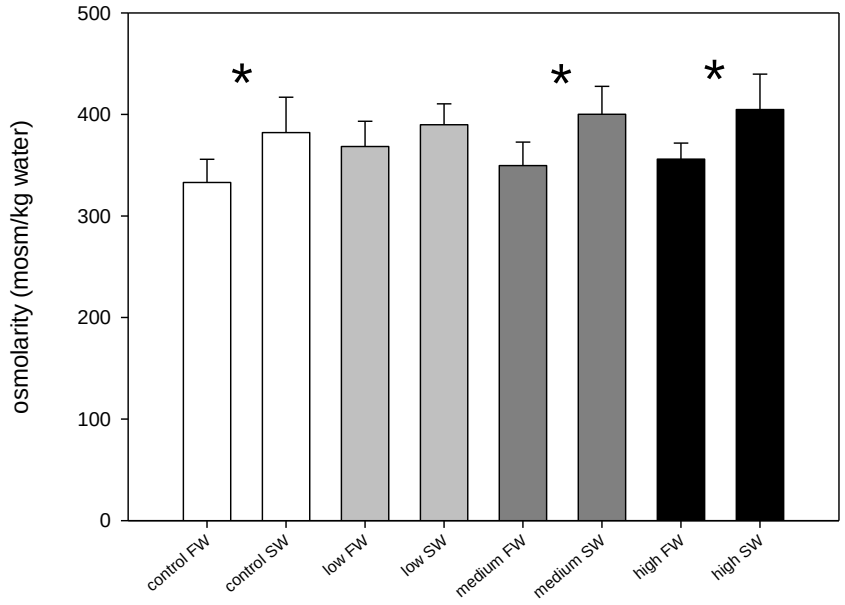


Figure 5.3 Plasma osmolarity in eels exposed to concentrations of 0.001 µg l-1(Low), 0.01 µg l-1 (Medium) and 0.05 µg l-1 (High) fenitrothion in freshwater (FW) and after the saltwater challenge (SW). The data represents mean ± SD of 7 eels per group. * p<0.05 between FW and SW groups.

The plasma osmolarity showed the expected significant increase in the control groups when the eels were moved from freshwater into saltwater (Figure 5.3). However, there were similar increases in the Medium and High exposed groups suggesting that fenitrothion has very little effect on the ability of the eels to osmoregulate in saltwater. A similar trend was shown in the plasma levels of Cl^- ions. The levels significantly increased in the control group and the groups exposed to fenitrothion (Figure 5.4).

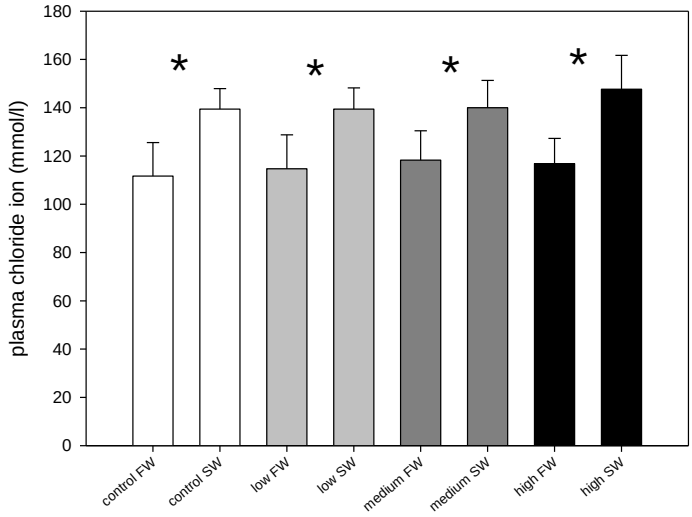


Figure 5.4 Plasma Cl- ion concentrations in eels exposed to concentrations of 0.001 µg l-1(Low), 0.01 µg l-1 (Medium) and 0.05 µg l-1 (High) fenitrothion in freshwater (FW) and after the saltwater challenge (SW). The data represents mean ± SD of 7 eels per group. * p<0.05 between FW and SW groups.

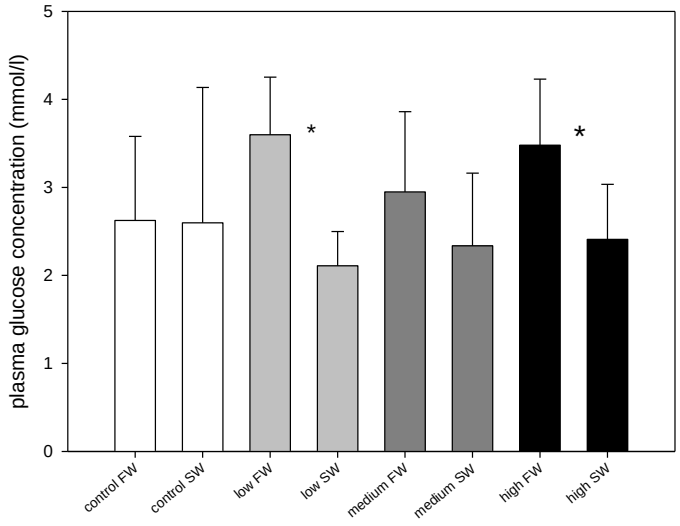


Figure 5.5 Plasma glucose concentrations in eels exposed to concentrations of 0.001 µg l-1(Low), 0.01 µg l-1 (Medium) and 0.05 µg l-1 (High) fenitrothion in

freshwater (FW) and after the saltwater challenge (SW). The data represents mean ± SD of 7 eels per group. * p<0.05 between FW and SW groups.

The glucose levels in the control groups did not change when the eels were moved from freshwater to the saltwater (Figure 5.5). However, in the Low and High exposed groups there were a significant decline in the concentrations of plasma glucose after transfer to saltwater. Generally, it would be expected that the glucose levels would decline in eels when moving to saltwater. In a similar study (Privitera et al., 2014) where eels were exposed to TBP (see Section 5.1.2), there was a significant decrease in the levels of plasma glucose in the control groups when transferred to saltwater, but no change in the levels of glucose in the group exposed to TBP. Glucose has a major role in the bioenergetics of animals, and carbohydrate metabolism appears to play a major role in the energy supply for osmoregulation. There are considered to be spatial and temporal relationships between the liver and osmoregulatory organs in partitioning the energy supply for ion regulatory mechanisms during changes in salinity (Tseng & Hwang, 2008). The decrease in the glucose levels in exposed fish may therefore have been the metabolic cost of osmoregulation by the eels in saltwater. However, it is not clear why there was not a similar decrease in glucose in the control fish, although this may just be due to high variability between the samples.

Disease status of experimental fish.

Many countries have started compiling data on the health status of eels in their water bodies. Objectives for these monitoring actions are diverse and there is a large amount of information collected by EU member countries. However, this information is widely scattered over Europe in agencies, institutes or universities. As there is a growing need to collect and report on data on the health status of the eel on international level, the Joint EIFAC/ICES Working Group on Eels initiated in September 2007 the setup of a European Eel Quality Database to collect recent data of contaminants and diseases over the distribution area of the eel.

The external screening of the 5 specimen sent to the Cefas Weymouth Laboratory (full report in Appendix 3) identified a number of lesions around the caudal peduncle and tail fin, although these were considered to be caused by the method of capture.

Internally, the only observation was that some fish had fatty deposits surrounding the heart. Overall, there did not appear to be any obvious problems with the general condition of the eels.

Examination of the eels noted the presence of the following parasites.

Anguillicola crassus (Nematoda) – present in the swim bladder

Paraquimperia tennerima (Nematoda) - present in the intestine

Acanthocephalus lucii (Acanthocephala) - present in the intestine

Pseudodactylogyrus sp. (Monogenea) – present in the gills.

The virology screening was undertaken using samples of spleen, kidney and brain from each fish and pooled. This was then inoculated onto the following cell lines BF-EP, EP-20, CH-15, FH-15 and FH-20's. The results indicated that the viruses VHS, IHN, IPN, SVC and eel rhabdovirus were not present in any of the eels. A further PCR for eel herpesvirus was also negative.

Overall, the health of the sample of eels from the River Stour was generally good although the presence of the parasite *Anguillicola crassus* is of concern. *Anguillicola crassus* is not native to British fish species and is classified by the Environment Agency as a Category 2 Parasite. *Anguillicola crassus* is considered to be the most aggressive fish parasite to have been introduced anywhere in the world. It was originally discovered in the swim bladder of the Japanese eel, *Anguilla japonica* in south-east Asia, where it is native, but was introduced to Europe in the 1980s when Japanese eels were imported to Europe for aquaculture (Kirk, 2003). Once introduced, *Anguillicola crassus* quickly infected adults, elvers and glass eels in wild populations of the European eel. The parasite lives in the swim bladder of eels, where it may do damage and reduce the eel's ability to maintain and adjust buoyancy. Recently, research has shown that infection by the parasite reduces the swimming speed (Sprengel & Lüchtenberg, 1991) and endurance of eels because the parasite is a 'metabolic burden' i.e. reduces the metabolic efficiency of the eel (Palstra et al., 2007). Aside from increasing the energy costs of swimming, this stress may also have a long-term effect because eels will be less able to gain and store fat during their time in freshwater. This in turn may affect the ability of the eels to successfully undertake

their oceanic migration and produce viable off spring. However, in the context of the present study it is unlikely that the parasite would have interacted with any effect shown by the pesticide fenitrothion.

In conclusion, the research indicates that exposure of the European eel to environmentally relevant concentration of fenitrothion in freshwater for a short period, does not compromise the eels' ability to successfully undergo the silvering process or their ability to osmoregulate as they enter the marine environment. However, eels may be exposed for longer periods in the wild to the pesticide and the impact of long term exposure is not known. In addition, the ability to subsequently survive in saltwater was only measured for a short period and the long term effect of this pesticide is not known. Finally, the present study has not examined other toxic effects that the pesticide may have on the eel such as reducing spawning success and modifying the bioenergetics of oceanic migration.

5.3. Silver eel and tributyl phosphate

5.3.1. Methods

Tributyl phosphate (TBP) is used as plasticizer and flame retardant and its details have been described in Chapter 3. This research examined the impact of freshwater exposure to TBP on migrating wild silver eels during their subsequent emigration from freshwater and into the coastal zone. The study used an integrated physiological and behavioural approach to the research and was undertaken in collaboration with DTUAqua (Denmark). European eels were tagged with miniature coded acoustic transmitters (see Section 2.7 for details) and exposed in the laboratory to an environmental concentration of TBP (Fries & Püttmann, 2003). Their subsequent movements were monitored as they migrated through a freshwater river and fjord and into the marine environment, using strategically positioned acoustic receivers. A laboratory-based study was also carried out on the morphology and physiological status of groups of eels exposed to TBP to determine whether the contaminant had a direct effect on the silvering process, osmoregulatory capability and survival of the fish once they had moved into saltwater.

In November 2010, 80 silver eels were captured in a modified Wolf trap situated at the Vestbirk hydropower station in the upper part of the River Gudenaa, Denmark (55°58′35.94″N, 9°42′2.53″E), and maintained in a holding pen at the catch site for 0–6 days. Fish were then brought to the Centre for Vildlaks in Randers, Denmark. The fish were then distributed between two of four identical indoors tanks (Figure 5.6) with a volume of 300 l and kept with a continuous freshwater flow (5.4–8.2 °C) and an artificial light – dark cycle of 8-h light – 16-h dark to represent natural conditions in the area. Fish were not fed whilst they were left to acclimatise for one week as silver eel are considered to cease feeding and show egression of the alimentary tract (Van Ginneken et al. 2007a).

Figure 5.6 Holding tanks for eel exposed to TBP.

Migratory behaviour of silver eels

At the end of the acclimatisation week, 40 fish were randomly selected by dip netting and tagged intraperitoneally with VC9 acoustic tags as described in Section 2.7. Tagged eels were then equally distributed over the two remaining tanks (control and exposed) in static oxygenated freshwater. Fish were allowed to recover from surgery for 2 days, before the fish were exposed to the treatment. The treatment consisted of an exposure to 0.5 µg l^{-1} of TBP (Sigma-Aldrich, Gillingham, Dorset, UK) for a period

of 5 days. The concentration of 0.5 μg l^{-1} was chosen to best represent the values reported from a limited number of water courses supporting eel populations (For rivers in Japan, Switzerland and Norway see WHO 1991; for rivers in the river Oder catchment see Fries & Püttmann, 2003). The duration of the experiment was short as the fish used in this experiment were at the end of the normal migration period for this river system (K. Aarestrup, pers. comm.), and as indicated in a previous study (Aarestrup et al., 2010), some eels can migrate downstream rapidly and take as little as 3 days to enter the Kattegat from the first acoustic listening station (Figure 5.7).

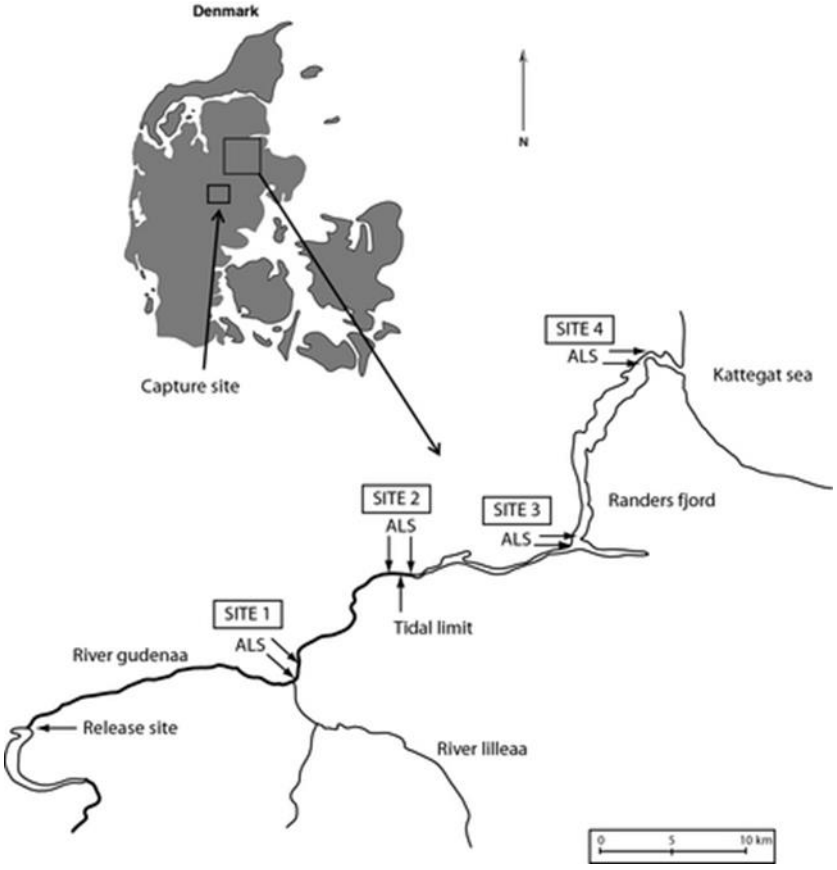


Figure 5.7 Capture and release sites for tagged silver eels and position of the eight pairs of acoustic receivers (ALS) at sites 1–4 along the River Gudenaa and Randers Fjord.

On the 19th November, at the end of the 5-day exposure period, all tagged fish were transported and released (10:00 am) at Tange hydropower station (Fig. 5.7 and 5.8), on the River Gudenaa, Denmark (coordinates 56° 21′ 18.34° N; 9° 36′ 14.76° E). The River Gudenaa (mean annual discharge of 32 m^3/s) is the major freshwater source to the narrow Randers Fjord (Figure 5.8). The Randers Fjord (30 km long) is principally

divided into two parts: a narrow inner section and a wider outer section, which exits into the Kattegat (Aarestrup et al., 2010). The salinity varies with water discharge in the River Gudenaa, but the fjord can generally be characterised as brackish, and salinity increases with depth and with increasing distance from the head of tide.

Figure 5.8 Tagged eels after exposure in laboratory condition are transported and released downstream of Tange Hydropower station.

Eight VR2 acoustic receivers (Vemco, Inc.) were deployed at four sites (Acoustic Listening Stations, ALS) in the river and fjord and left until May 2011 (Aarestrup et al., 2010). The receivers recorded and stored the unique transmitter code and time (to the nearest second) of individual fish when a tagged fish moved within range. Two receivers were positioned at each site, separated by a few hundred metres in a longitudinal direction, to confirm detection and swimming direction. A previous study has indicated that detection ranges of each receiver varied between 130-400 m during range tests, the width of the river or fjord adjacent to the receivers varied between 50-240 m (Aarestrup et al., 2010), and therefore, the receivers were positioned to maximise detection rates of the tagged eels. However, changes in salinity, speed of movement of eels and the levels of sediment within the water column can all affect the detection rates of acoustic tags, and so, some eels may not have been detected by all receivers. The distances from the release site to each of the four receiver sites were 22, 37, 49.2 and 65.6 km.

The movement of the eels as they were initially detected by the acoustic receivers was analysed using vector analysis and the software package Oriana 4 (Kovach, 2011). The data sets were specifically tested to show whether the movement of the eels was random with respect to time of day and state of tide using the Rayleigh test (*r* value)

(Batschelet, 1981). The mean values of time of day, together with the *r* values have been calculated for each receiver. The value of *r* is a measure of angular dispersion and in this respect can be used to determine whether the movement of the fish are directed in relation to time of day. An *r* value of 0 means uniform dispersion, whilst a value of 1 means complete concentration in one direction or time of day/tidal cycle.

Saltwater challenge test on silver eels.

The 40 fish that were not tagged were distributed equally between two tanks containing freshwater at a temperature ranging from 5.4 to 8.2 °C, and one of the two was then exposed for 5 days to a concentration of 0.5 μg l^{-1} TBP. At the end of the 5 days exposure, 10 fish from each tank were sampled to allow tissue and blood collection for physiological measurements. The remaining fish were left in the tanks, and the freshwater replaced with full strength (35 ‰) artificial saltwater (Red Sea Coral Pro salt, 38.2 g l^{-1}). Water temperature during the saltwater challenge was between 5.4 and 6.8 °C. Fish were left in saltwater for 3 days and monitored daily to determine any sign of distressed behaviour (e.g., erratic swimming movements or unresponsiveness) and survival. At the end of the saltwater challenge, all surviving fish were sampled for the same parameters as in the previous 20 fish. A 3-day saltwater challenge was chosen as previous studies have indicated that a number of the physiological parameters measured in this study undergo significant changes within this period. For instance, plasma Na^+ and Cl^- levels peak 2–3 days after the introduction of adult eels into saltwater (Bornancin & De Renzis, 1972; Kirsch & Mayer-Gostan, 1973; Ho & Chan, 1980). However, although Na^+/K^+ ATPase takes longer to reach peak values (e.g., 7 days or more) (Bornancin & De Renzis, 1972; Ho & Chan, 1980), the increase in activity is initiated immediately after saltwater entry and as such can be used to compare the impact of the contaminant against the appropriate control.

The morphological and physiological biomarkers that were measured to determine the silvering stage of the eel and its ability to osmoregulate within saline conditions have been detailed in the section on fenitrothion.

5.3.2. Results and discussion

Migratory behaviour of silver eels

Eels were assigned randomly to control and exposed group. Fish length (control 64.05 ± 0.9 cm; exposed 63.6 ± 1.07 cm, mean ± SEM – t = 0.320, d.f. 38 P = 0.750) and fish weight (control 465.05 ± 25.59 g; exposed 481.9 ± 24.93 g, mean ± SEM – t = -0.472, d.f. 38 P = 0.646) did not differ significantly between the two groups.

The times that the two groups of eels were detected at each of the first 3 receiver sites are shown in Table 5.3. The data from the two receivers at each site has been pooled.

Table 5.3 The downstream movements of the two groups of tagged eels at each of the 3 receiver sites on the River Gudenaa in relation to the time of day. The mean times that eels were recorded passing each site have been calculated from the mean vectors (Batschelet, 1981). The "*r*" values provide a measure of randomness of movement in respect to time calculated using the Rayleigh test. The value n is the total number of eel movements through each of the respective sites.

	Site 1	Site 1	Site 2	Site 2	Site 3	Site 3
	Control	Exposed	Control	Exposed	Control	Exposed
Mean time	16:08	17:05	23:04	23:37	Random movement	Random movement
r value	0.726	0.759	0.891	0.693	0.441	0.392
P	< 0.0001	< 0.0001	< 0.0001	< 0.001	= 0.023	= 0.116
N	32	32	18	18	19	14

One of the two most seaward receivers at site 4 was lost before the data could be downloaded and the number of detections on the remaining receiver was insufficient to complete any analysis of the movement of the eels in relation to the time of day. Therefore, this data has not been included. The movement of both groups of eels in

the freshwater section of the River Gudenaa (Site 1) was principally nocturnal (Figure 5.9).

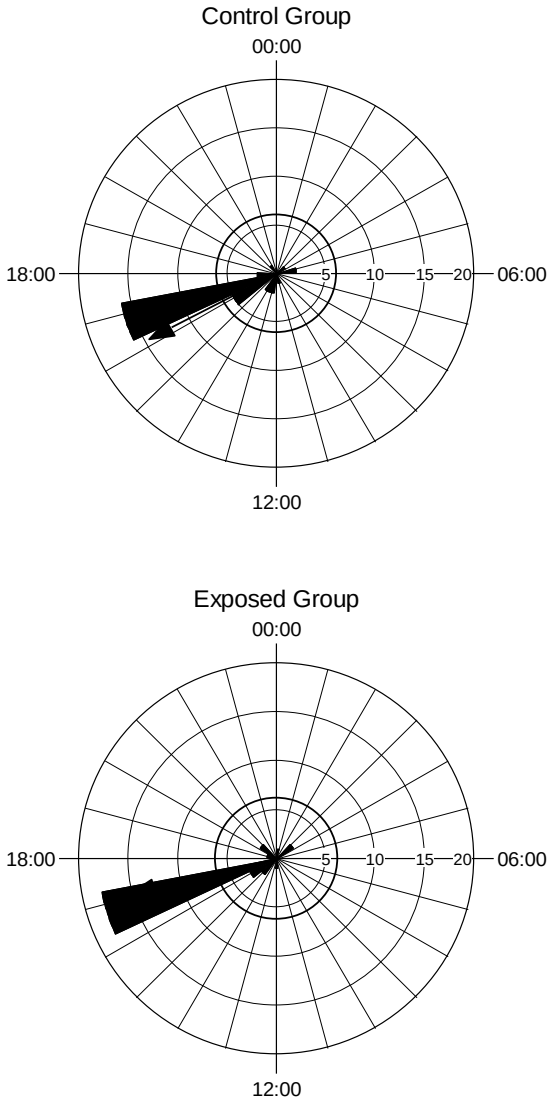


Figure 5.9 Histogram showing the time of day that the two groups of eels were detected migrating downstream at the receivers located at Site 1. The histogram provides an indication of the number of eels at each time. The arrow represents the mean time of detection calculated by vector analysis and its length represents the *r* value calculated using the Rayleigh test (Batschelet, 1981).

There was no difference in the movements of the two groups of eels with mean times of 16:08 (Control) and 17:05 (Exposed) (Mann-Whitney Rank Sum Test, T = 1064.500, P = 0.322) Figure 5.9.

The movement of the eels past the tidal limit of the river (Site 2) continued to be directed with respect to time of day with fish moving at mean times of 23:04 (Control) and 23:37 (Exposed) (Table 5.3). However, in the middle of Randers Fjord, the movement of both groups of eels became random with respect to time of day and they were recorded moving seaward during both the day and night (Table 5.3).

There were no significant differences between the two groups of eels in terms of the migration rates from release to each of the first 3 receiver sites. The mean time taken for the two groups to reach the first receiver position was control 6.93 ± 1.09 h; exposed 12.92 ± 5.54 h (mean ± SE – Mann-Whitney Rank Sum T=225.500, P = 0.513). Migration rates from the point of release to the middle of Randers Fjord were also not significantly different between the two groups, and the mean time for each group to reach the receivers was Control 95.48 ± 45.65 h; exposed 117.55 ± 75.39 h (mean ± SE Mann-Whitney Rank Sum T = 58, P = 0.867).

Saltwater challenge test on silver eels.

The results of the saltwater challenge test indicated there were no mortalities in the exposed or control groups whilst maintained in freshwater or when they were introduced to saltwater. Throughout the duration of the experiment, all fish showed no signs of distress or abnormal behaviours. Assessment of the swim bladder parasite *Anguillicola* indicated that 46% of the eels had no parasite present while the remaining 54% had some level of parasite infestation. The measured morphological parameters (Table 5.4) indicated no significant difference (2-way ANOVA) between control and exposed fish or between fish within each group exposed to the saltwater challenge or not.

Table 5.4 Morphological parameters measured after freshwater exposure and after saltwater challenge.

Morphological parameters	Freshwater		Saltwater	
	Mean ± S.E.M.		Mean ± S.E.M.	
	Control	Exposed	Control	Exposed
weight (g)	305.5 ± 27.49	289.5 ± 14.0	354.7 ± 21.0	318.6 ± 23.0
length (cm)	55.75 ± 1.44	55.25 ± 1.09	56.35 ± 1.67	57.4 ± 1.21
fin length (mm)	28.03 ± 0.56	27.43 ± 0.78	26.67 ± 0.76	27.26 ± 0.82
eye index	6.71 ± 0.19	7.2 ± 0.28	6.85 ± 0.44	6.7 ± 0.33
G.S.I	1.12 ± 0.08	1.31 ± 0.08	1.26 ± 0.18	1.03 ± 0.08
H.S.I.	1.39 ± 0.07	1.48 ± 0.07	1.58 ± 0.1	1.47 ± 0.1

Eels in all groups were of similar size; however, their condition factor (CF) was significantly different (Figure 5.10) as control eels after saltwater challenge had a higher CF than all other groups. This difference is probably due to differences in the fat stores measured as a percentage by means of an ultrasound fat metre (Distell, fish Fatmeter). Fat content (Figure 5.10) was significantly lower in control fish after saltwater challenge. Fish were not fed throughout the experiment, and so, any differences in fat content between the two groups probably existed prior to the exposure to the contaminant.

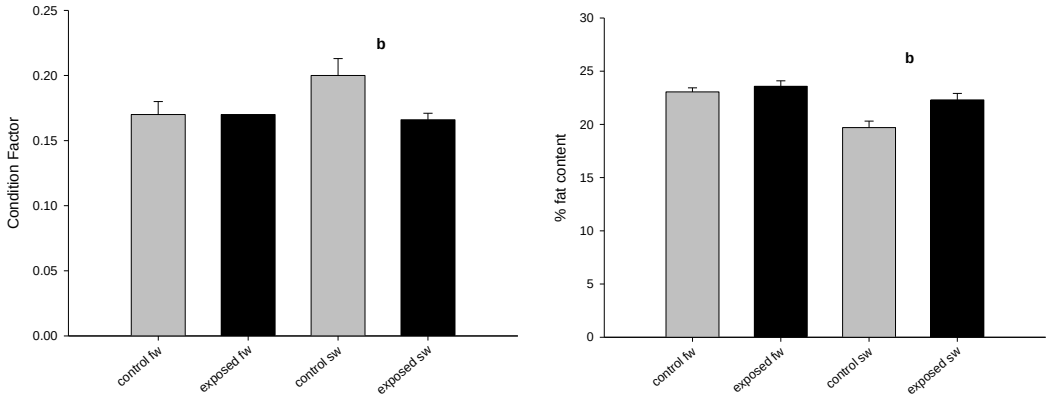


Figure 5.10 CF and fat content in silver eels. Significant difference between treatments is indicated by b (2-way ANOVA, CF P=0.031; fat content P=0.07).

Results of plasma analyses demonstrated a significantly higher osmolarity (Figure 5.11) for fish in saltwater compared with freshwater, which is to be expected in fish moving from a freshwater to a marine environment.

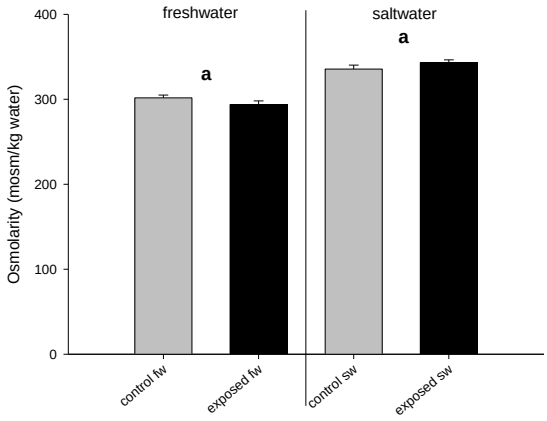


Figure 5.11 Plasma osmolarity in silver eels exposed to TBP in freshwater and then transferred to saltwater. There is a statistically significant difference (2-way ANOVA a, P<0.001) between osmolarity measured in fish while in freshwater and after transfer to saltwater.

Plasma concentrations of potassium and calcium did not differ between the exposed and the control groups both in freshwater or saltwater (Figure 5.12).

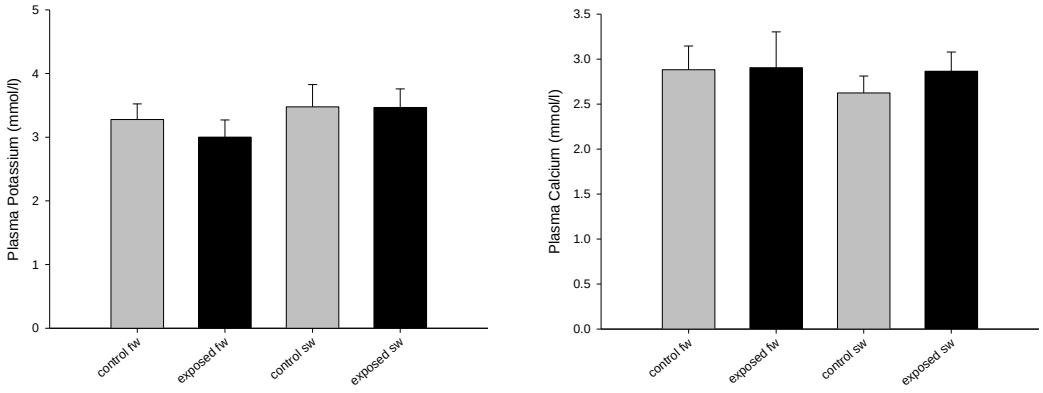


Figure 5.12 Plasma concentration of potassium and calcium.

However, concentrations of sodium and chloride were affected by exposure to TBP. The concentrations of both ions were lower in the exposed compared with the control groups in freshwater and when moved to the saltwater treatment (Figure 5.13). However, there was the expected increase in both ions when the eels were transferred to saltwater.

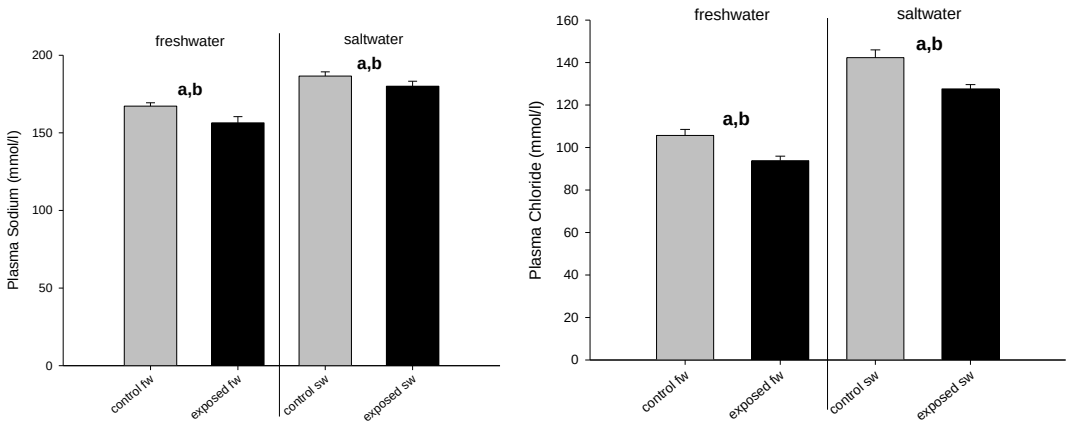


Figure 5.13 Concentrations of plasma sodium and chloride. Significantly different concentrations between freshwater and saltwater are indicated by a (for both sodium and chloride P<0.001) and significant differences between treatments are indicated by b (sodium P=0.009 and chloride P<0.001).

In addition, exposure to TBP did not result in the expected decrease in glucose concentration when the eels were moved into saltwater as demonstrated by the control group (Figure 5.14).

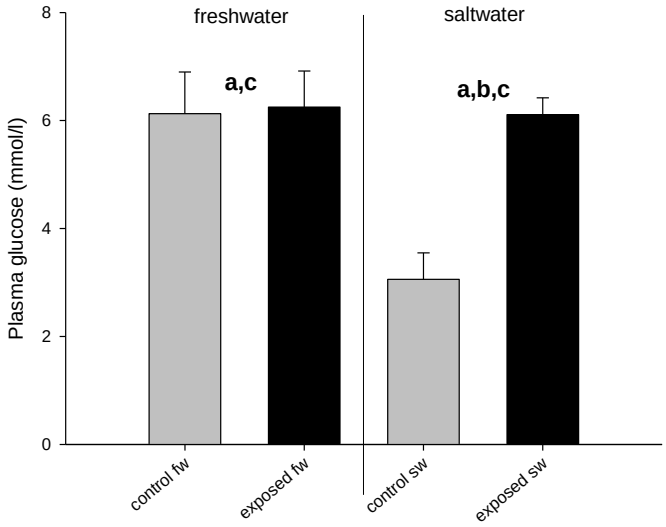


Figure 5.14 Plasma concentrations of glucose. Difference between freshwater and saltwater a P= 0.011; difference between treatments b P=0.01 and interaction between treatment and salinity challenge c P=0.017.

Eels exposed to TBP did not show any differences in gill Na^+/K^+ ATPase activity in either fresh or saltwater, but there was significant impact of TBP on kidney Na^+/K^+ ATPase activity (Figure 5.15). There was not the expected decrease in enzyme activity in exposed fish after transfer to saltwater.

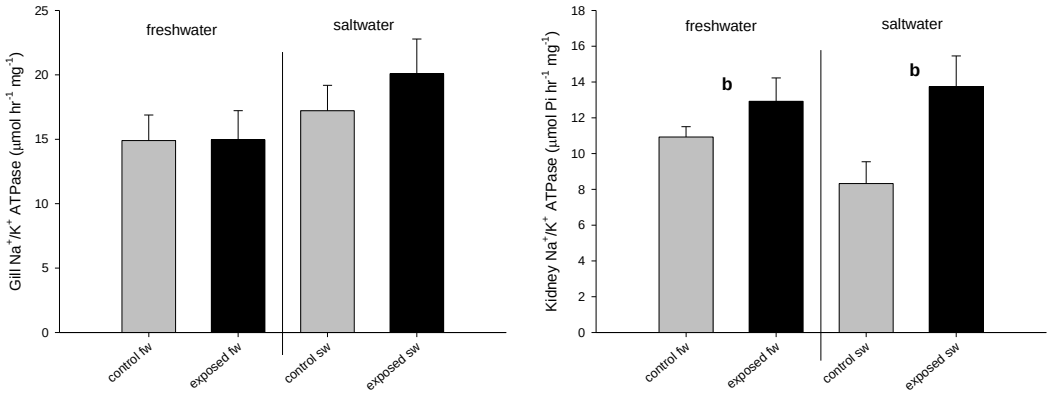


Figure 5.15 Gill and kidney ATPase. Significant difference between treatments is indicated by b P=0.006.

Overall, the results of the study indicated that exposure to TBP for a short period had no significant impact on the migratory behaviour or migratory success of eels moving through the lower section of the River Gudenaa and Randers Fjord. However, only 12% of the tagged eels were detected at the receiver site closest to the exit into the Kattegat Sea. In a study in the River Meuse, Belgium (Verbiest et al., 2012), 15% of the tagged eels that migrated within freshwater entered the North Sea. The suggested high mortality was considered to be the result of hydroelectric power stations, exploitation or predation although a number of the fish also remained within freshwater and did not migrate further. A previous study in Randers Fjord demonstrated that 40% of eels were detected migrating through the study area (Aarestrup et al., 2010) and that the likely cause of the low detection rate of tagged fish was related to fishing mortality within the fjord. Whilst fishing pressure has probably played an important role in the low detection rate observed in this study, it is also worth noting that there are other possible causes for the low detection rates of tagged eels other than fishing mortality. The procedures used in the capture and tagging of the eels may have an impact on their subsequent behaviour and migration through the study area (Jepsen et al., 2002). Fish are known to expel tags that have been surgically implanted into the body cavity (Moore et al., 1990), although Winter et al. (2005) studied tagging effects in European silver eels and recorded no transmitter expulsion or mortality related to handling or tagging of the fish. Tags may also have failed due to technical problems and as such migrating eels would not be detected as they moved through the study area. In addition, changes in salinity, swimming speed

and the levels of sediment within the water column can all affect the detection rates of acoustic tags, and so, it is possible that some eels may not have been detected by all receivers. In the present study, it should be noted that one of the two receivers at the exit of the fjord was lost at some point during the study and reduced the detection rates of migrating eels. It is possible that fish were also not detected because they remained close to the release site after tagging and either did not migrate or only migrated later after the study period. Although the eels were trapped during what is considered to be the main migration period for the population in the River Gudenaa, the eye index of the eels, an indicator of silvering in eels, was relatively low compared with other studies (Durif et al., 2005; Van Ginneken et al., 2007a) but still above the threshold of 6.5 which Pankhurst (1982) suggested to be associated with the onset of sexual maturation; however, the GSI measured in this experiment is in line with values typical of silver eels (Van Ginneken et al., 2007a). Eels that were not totally pre-adapted to saline conditions may not have migrated immediately into the sea but resided for a longer period in freshwater (as reported by Feunteun et al., 2000). Migration through the estuary in the present study was also predominantly nocturnal as previously described by Aarestrup et al. (2010) although the seaward migration of the eels in the present study was significantly faster. The time taken for the eels to reach the most seaward receivers in the fjord from the point of release was 138 ± 32:41 h (mean ± SD) compared with 742 ± 375 h (mean ± SD) as reported by Aarestrup et al. (2010). The differences in migration rates could be attributed to possible differences in the migratory behaviour of the eels as a result of differences in release date (November vs. September–October), development (silver eels in the previous study by Aarestrup et al., 2010 were heavier than the eels used in the present study), handling and tagging or differences in the environmental conditions within the river during the two studies.

Although the exposure of eels in freshwater to TBP resulted in certain parameters related to osmoregulation being affected, it did not affect the survival of the fish. Eels were physiologically stressed by exposure to the contaminant as shown by significantly reduced plasma sodium and chloride ion concentrations. Plasma chloride concentrations have been shown to decrease when freshwater fish are stressed (Nomura et al., 2009). There were no differences in the other plasma ions between the two treatment groups in freshwater, and there were no significant differences in plasma osmolarity. In addition, there were no significant differences in HSI or GSI between

the two groups. Esteve et al. (2012), demonstrated that eels that had bioaccumulated a range of metals had a reduced HSI and a lower fitness. The similarity in the HSI (and probably GSI) measured in the present study possibly reflects the low bioaccumulation (WHO, 1991) of TBP in fish or the short period of exposure of the eels to the contaminant.

However, when transferred to saltwater, there were additional physiological differences in the eels that had been exposed to TBP, although there were no mortalities in either of the two groups. Generally, fish in freshwater need to eliminate excess water gained osmotically via their kidneys but conserve ions, but the reverse is true in saltwater (Rankin, 2009; Tang et al., 2012). The expected decrease in kidney Na^+/K^+ ATPase activity was evident in the control group, but levels in the exposed group were similar to those measured in freshwater. These effects may help explain the significant declines in plasma sodium in exposed eels, but not those in chloride, nor the lack of differences in potassium or calcium. However, there was no impact of TBP exposure on gill Na^+/K^+ ATPase as seen in eels after exposure to the heavy metal cadmium (Lionetto et al., 1998) nor was there the expected increase in gill Na^+/K^+ ATPase activity in the control group after transfer to saltwater (Tang et al., 2012). TBP is readily assimilated by fish, but after metabolic transformation in the liver, hydroxylated butyl moieties are eliminated via the kidneys (Sasaki et al., 1982; WHO, 1991). The activation of the metabolic pathways involved in TBP degradation, transport and elimination could help explain why plasma glucose was elevated in exposed eels and why TBP exposure affected kidney but not gill Na^+/K^+ ATPase and affected plasma sodium and chloride but not calcium and potassium. The plasma levels of sodium and chloride ions increased when the eels were transferred to saltwater (Bornancin & De Renzis, 1972) and were similar to those recorded by Kirsch (1972). In eels, plasma level of sodium normally rapidly increase after introduction in saltwater but decreases back to the same level as while in freshwater within 7 days (Ho & Chan, 1980). Plasma chloride concentration also increases after entry into saltwater and remains high (Kirsch & Mayer-Gostan, 1973). Although there was an increase in both ions in the exposed group, the levels were still significantly lower than in the controls, again suggesting physiological stress as a result of contaminant exposure.

There was a significant decrease in the levels of plasma glucose in the control group when transferred to saltwater, but no change in the levels of glucose in the group exposed to TBP. Glucose has a major role in the bioenergetics of animals, and carbohydrate metabolism appears to play a major role in the energy supply for osmoregulation. There are considered to be spatial and temporal relationships between the liver and osmoregulatory organs in partitioning the energy supply for ion regulatory mechanisms during changes in salinity (Tseng & Hwang, 2008). The decrease in the glucose levels in control fish may therefore have been the metabolic cost of osmoregulation by the eels in saltwater. However, it is not clear why there was not a similar decrease in glucose in those fish exposed to TBP, although the high levels of glucose may be related to the stress of exposure to the contaminant. In suboptimum or stressful conditions (e.g., exposure to poor water quality/pollution), the chromaffin cells in fish release catecholamine hormones, adrenaline and noradrenaline towards blood circulation (Reid et al.,1998). Those stress hormones in conjunction with cortisol mobilise and elevate glucose production in fish through gluconeogenesis and glycogenolysis pathways (Iwama et al., 1999) to cope with the energy demand produced by the stressor for the 'fight-of-flight' reaction. This glucose production is mostly mediated by the action of cortisol which stimulates liver gluconeogenesis and also halts peripheral sugar uptake (Wedemeyer et al., 1990). However, cortisol has also been identified as a seawater-adapting hormone in a large number of teleost species (McCormick, 2001) and to be implicated in osmoregulation, regulating Na^+, K^+-ATPase activities which are prime determinants of osmoregulatory capacity (Mancera & McCormick, 2007). In eels, there is a transitory increase in plasma levels of cortisol on transfer to saltwater (Forrest et al., 1973). The increased levels of glucose in the exposed fish may therefore represent the additional glucose produced by an increase in stress related cortisol which is not utilised metabolically for osmoregulation.

The physiological changes observed in the eels after exposure to TBP did not appear to have had a significant impact on the short-term migration patterns observed within the River Gudenaa and fjord. This is in contrast to similar studies on Atlantic salmon smolts which also undergo a physiological transformation during the transition from the fresh to marine environment but have been shown to be sensitive to exposure to freshwater contaminants (Waring & Moore, 2004; Lower & Moore, 2007). Although

certain contaminants can affect the physiological processes involved in salmonid smoltification, where there is a period to allow recovery from the exposure to the chemical, the subsequent migratory behaviour of the fish may not be significantly affected (Moore et al., 2008). Therefore, the period between exposure in freshwater and entry into the sea may be critical in terms of whether migration and survival in the marine environment are compromised. Contaminants that occur within estuaries and which the eels are exposed to immediately prior to saltwater entry may be more of a concern than those occurring in areas of the freshwater environment where there is a significant period between exposure and the migration of the eels into the sea (Moore et al., 2008).

The results of this study suggest that exposure to the contaminant in freshwater does modify the physiological processes involved in osmoregulation once the fish have migrated into seawater. In terms of the life cycle of the eel, the freshwater and marine environments cannot be considered in isolation, and the conditions experienced by the eels in rivers and lakes may have a direct impact on their subsequent physiology and survival in the marine environment (Waring & Moore, 2004). In the present study, the eels were exposed to a short contaminant exposure period. However, the freshwater stage of the eel may last for a number of years (Tesch, 2003) and exposure to single and suites of contaminants for long periods may have a more significant impact on salt water survival. Further, it is not known how the physiological perturbations observed in the present study may affect the extensive marine migration of the eel or in terms of its subsequent reproductive success. Eels are known to spend long periods residing within sediments in rivers and lakes and further studies on the potential effects resulting from contaminated sediments on eel physiology, and migratory behaviour is therefore required to examine the long-term impacts of contaminant exposure on marine survival in the European eel.

5.4. Silver eels and a pesticide mixture

A similar integrated physiological and behavioural study was carried out to investigate the impact of a mixture of pesticides on the physiology and the downstream migratory behaviour of silver eels during their transition from the freshwater to the marine environment. The River Avon in Hampshire was chosen as the study site for the behaviour study as the catchment (Figure 5.16) is largely dominated by intensive farming and unimproved grasslands (Jarvie et al., 2008) and the river has significant levels of agricultural pesticides. These pesticides were the basis for the laboratory and field based studies. The River Avon is located in the south of England, it starts at Pewsey and runs to Christchurch in Dorset. The overall Avon catchment area is about 1750 km^2, and only 2% of the catchment is urbanised. The Avon catchment is characterised by open chalk downland with steep scarp slopes, sheltered valleys, chalk hills, ridges and limestone plateaux. These significant variations in the topography have a strong influence on the rivers' response to rainfall (Environment Agency, 2012). The rivers of the Avon catchment are largely spring-fed, which provides relatively stable flow throughout the year, although hydrological differences are observed on some of the tributaries, reflecting their different geologies.

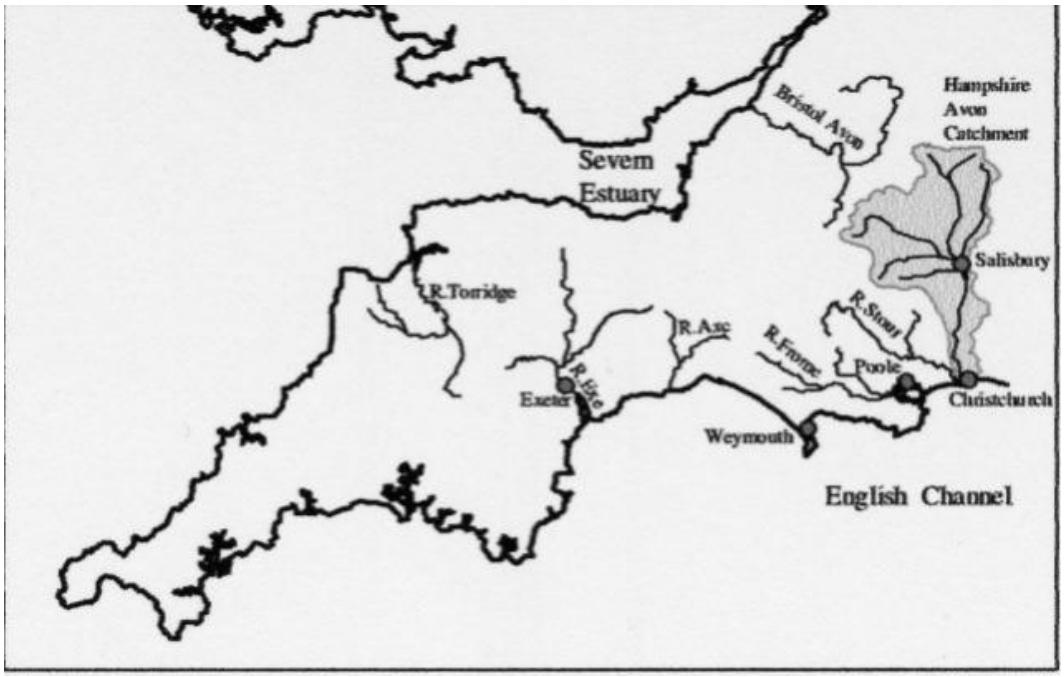


Figure 5.16 The Hampshire Avon catchment (Environment Agency).

In addition to the behaviour of migrating eels, the impact of the pesticide mixture on their metabolism was also investigated in order to determine whether there was an effect on the energy levels in eels required to complete a successful spawning migration. Currently there is very little information on the potential metabolic cost of exposure to contaminants in the European eel. The detrimental effects of pollution on fitness and fecundity have been suggested by a number of other studies to be factors causing the decline of the eel (see Robinet & Feunteun 2002 for a summary). Any contaminant that results in a high metabolic cost to the eel as a result of reduced lipid levels or excess energy requirements to excrete or store these compounds may reduce the migratory and/or spawning success in eels. Therefore, a laboratory based study was undertaken to examine the metabolic cost of exposure to pesticides as determined by changes to the individual standard metabolic rate (SMR) and routine metabolic rate (RMR) of eels.

The pesticides were chosen as they are commonly used in agriculture in England and are detected in rivers mainly during the winter months when the silver eels are migrating out to the marine environment. The concentrations selected represented environmental levels that are routinely monitored by the Environment Agency.

Pendimethalin [(N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine), CAS Nr 40487-42-1; Figure 5.17] is a dinitroaniline selective herbicide that inhibits the steps in plant cell division responsible for chromosome separation and cell wall formation (Strandberg & Scott-Fordsmand, 2004).

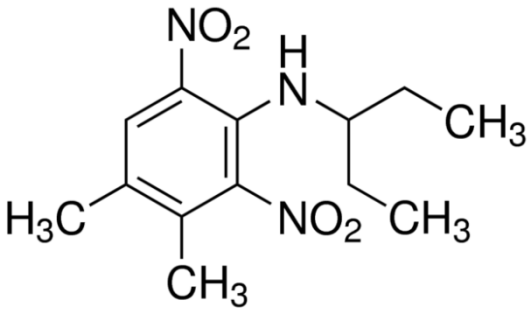


Figure 5.17 Pendimethalin chemical structure.

It is applied to crops such as cereals before emergence or added to soil before seeding as it inhibits the development of roots and shoots in seedlings. Pendimethalin is stable

and like its metabolites tends to bind to soil particles reducing the potential for leaching (WHO, 2003a). It has a low affinity for the water compartment with a solubility of 0.3 mg l^{-1} but especially in anaerobic conditions, polar metabolites are formed and can then reach ground and surface waters (WHO, 2003a). Its half-life in soil ranges from a few days to several months (Strandberg & Scott-Fordsmand, 2004) and it mainly enters surface waters as a run off after application. Under normal conditions the concentration of pendimethalin recorded in freshwater is up to 0.1µg l^{-1} with occasional peaks up to 6 µg l^{-1} been recorded in connection with surface run off after heavy rains (Strandberg & Scott-Fordsmand, 2004). In animals, pendimethalin appears to be poorly absorbed and rapidly excreted and the maximum tissue concentrations tend to be found in liver and kidneys. The reported 96-hour LC_{50} in rainbow trout is 138 µg l^{-1}, in bluegill sunfish is 199 µg l^{-1} and in channel catfish is 420 µg l^{-1} (Kidd & James, 1991).

Chlortoluron (3-(3-chloro-p-tolyl)-1,1 dimethylurea, CAS Nr 15545-48-9; Figure 5.18) is a pre- or early post-emergence herbicide used extensively to control annual grasses and broad-leaved weeds in winter cereals.

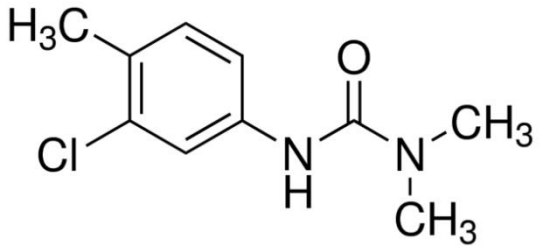


Figure 5.18 Chlortoluron chemical structure.

Its mode of action is to inhibit the photosynthetic electron transfer – PSII (Uno et al., 2011). It is slowly degraded in water and it is quite persistent (WHO, 2003b) with a half-life of several months. It is slightly mobile in soil and likely to reach surface waters after application. It has been detected in waters in the UK at concentrations ranging from 0.4 µg l^{-1} to 0.6 µg l^{-1} (WHO, 2003b).

Flusilazole [bis(4-fluorophenyl) (methyl)(1*H*-1,2,4-triazol-1-ylmethyl)silane, CAS Nr 85509-19-9; Figure 5.19] is a broad spectrum, synthetic triazole fungicide used to prevent and cure fungal disease in agriculture, horticulture and viticulture (Roberts & Hotson, 1999).

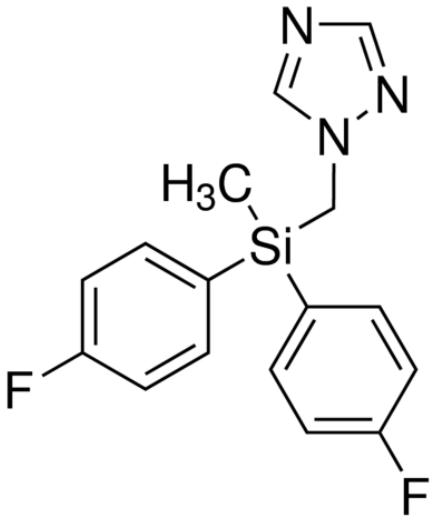


Figure 5.19 Flusilazole chemical structure.

It has low water solubility and it is categorized by the World Health Organization as "slightly toxic" (FAO, 2002). The LD_{50} in rat is 674 mg kg^{-1} suggesting a slight toxicity to mammals (Ozakca & Silah, 2013) and the LC_{50} for rainbow trout is 1.2 mg l^{-1} (FAO, 2002). Water concentration around agricultural settings was up to 0.07µg l^{-1} (EA 2005). In April 2013 the European Court of Justice withdrew the authorization to use flusilazole and starting from October 2013 (HSE, 2013) flusilazole products were withdrawn from the market for sale and supply while storage and use may continue until October 2014.

Copper oxychloride (CAS Nr 1332-40-7; Figure 5.20) is used mainly as a preventive fungicide sprayed directly on to crops (Maboeta et al., 2003) and applications can be repeated several times during the season (Krause et al., 1996).

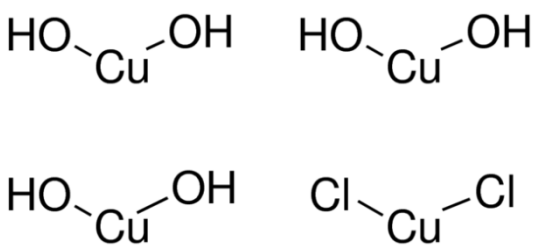


Figure 5.20 Copper oxychloride chemical structure.

Copper is an essential trace element for all biota as it is incorporated in a variety of enzymes and structural proteins. However, if organisms are over-exposed to copper physiological mechanisms that control homeostasis are affected and toxicity become evident due to the negative effects of copper on structure and function of proteins and molecules like the DNA (WHO, 1998). Copper oxychloride is water soluble but its bioavailability and more in general copper bioavailability in water can vary considerably according to the pH, absorption to particles or complexation with organic matters (de Oliveira-Filho et al., 2004). Its 96 hr LC_{50} for the zebrafish is 0.152 mg l^{-1} (de Oliveira-Filho et al., 2004).

Metaldehyde (2,4,6,8-tetramethyl-1,3,5,7-tetraoxacyclooctane, CAS Nr 108-62-3) is applied to crops and can find its way into drains and watercourses either directly during application or as a result of run-off during high or prolonged rainfall events (Water UK, 2013).

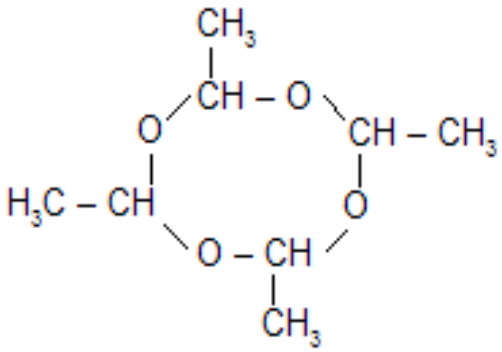


Figure 5.21 Metaldehyde chemical structure.

It is highly mobile in soils, and is generally stable to abiotic degradation mechanisms such as hydrolysis and photolysis. Metaldehyde is primarily dissipated from soils through biodegradation under aerobic conditions, with a half-life of approximately 2 months. Under anaerobic conditions, the half-life of metaldehyde is higher than 200 days. It has a low vapor pressure and therefore volatilization from soils and water surfaces will not be an important transport process (EPA, 2006). The results of acute toxicity studies with rainbow trout show that metaldehyde is slightly toxic to freshwater fish on an acute basis with a 96 hr LC_{50} of 69 mg l^{-1} (EPA, 2006). In toxicity tests carried out with another freshwater fish, the Nile tilapia, the 96 hr LC_{50} was 251.24 mg l^{-1} (Keratethaweesuk et al., 2013). Some water companies have recently been finding traces of metaldehyde in the raw water they abstract from rivers or reservoirs and treat to produce drinking water. These concentrations are extremely low – the highest being around 1µg l^{-1} and mostly much lower. However, the concentrations are above the European and UK standards for pesticides in drinking water set at 0.1µg l^{-1} (Environment Agency, 2011). It is however a seasonal issue, with levels increasing mainly in the autumn, when metaldehyde is applied to crops (Water UK, 2011). Current drinking water treatment methods designed to remove a range of pesticides are not effective at completely removing metaldehyde from water and there have been occasions when trace concentrations of metaldehyde have been detected in treated drinking water (Water UK, 2013).

Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate, CAS Nr 2921-88-2; Figure 5.22) is a broad spectrum organophosphate insecticide currently on the market and widely used in agriculture and in the home environment.

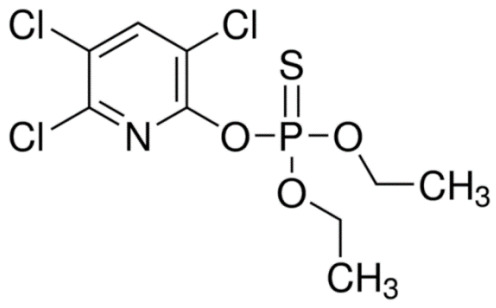


Figure 5.22 Chlorpyrifos chemical structure.

It is relatively persistent and has a half-life of 29 to 74 days (Racke, 1993). It is a very potent acetylcholinesterase (AChE) inhibitor (Amaroli et al., 2013), highly toxic to fish and it has been measured in surface waters at levels of 0.01-1.95 µg l^{-1} (Cerejeira et al., 2003; Palma et al 2009). The 96 hour LC_{50} for medaka is 0.12 mg l^{-1} (Khalil et al., 2013) and for rainbow trout is 7.1µg l^{-1} (Johnson & Finley, 1980).

5.4.1. Methods

Migratory behaviour of silver eels

In November 2012, 40 silver eels were trapped on the River Avon (Hampshire) and transported to the Cefas Lowestoft Laboratory. The eels were equally distributed into 4 identical tanks (volume of 550 l) and maintained with a continuous freshwater flow (water temperature varying between 4-10 °C) and a naturally simulated photoperiod. The eels were left to acclimatise for 2 weeks. At the end of the acclimatisation period eels were randomly netted, surgically implanted with acoustic tags as described in Section 2.8 and equally distributed over 4 identical tanks (same tanks, location and conditions as the acclimation tanks) to recover undisturbed for 7 days. At the end of recovery, the tanks were attributed to either a control or an exposed group (in duplicate). The exposed group was maintained for 5 days in a static freshwater system containing the pesticide mixture shown in Table 5.5.

Table 5.5 Mixture of pesticides.

Group	Compound	Concentration µg l^{-1}
Herbicide	pendimethalin	0.8
Herbicide	chlorotoluron	0.5
Fungicide	flusilazole	0.01
Fungicide	copper oxychloride	1
molluschicide	Metaldehyde	0.2
insecticide	chlorpyrifos	0.05

At the end of the exposure period the eels were transported back to the River Avon and released close to where they were trapped 4.5 km above the head of tide. Their subsequent movements were monitored using 10 strategically placed Vemco VR2W acoustic receivers located downstream from the release point and until the exit of the estuary in Christchurch Harbour (Figure 5.23).

Figure 5.23 Map showing the positions of the 10 VR2W acoustic receivers within the River Avon and Christchurch harbour. The arrow indicates where the tagged eels were released.

The movement of the eels as they were initially detected by the acoustic receivers was analysed using vector analysis as described in section 5.3.1. The data sets were specifically tested to show whether the movement of the eels was random with respect to time of day and state of tide using the Rayleigh test (*r* value) (Batschelet 1981).

In Christchurch Harbour there is a double high water on each tide and as a result there are generally 4 high waters every 25 hours. On full spring tides there is an approximately 4-hour flood from Low Water (LW) to the first HighWater (HW), followed by a lower second HW between 2 and 3 hours later. On neap tides there is an approximately 8-hour flood from LW followed by a 4 – 5 hours ebb. The first HW at Christchurch Harbour has been used as a reference time for all tidal cycles.

Respirometry and physiology of silver eels.

A further 16 eels obtained at the same time and location as the one above once brought to the Cefas Lowestoft Laboratory were tagged with PIT tags for individual recognition purposes. To allow insertion of PIT tags the fish were anaesthetized as described for acoustic tag implantation and a 2 mm incision was made on the abdominal side as described for acoustic tags. The PIT tag was inserted in the body cavity and the small incision was covered with a mixture of 50:50 orahesive and amoxicillin. This was enough to maintain tag retention (100% over the course of this study). Once tagged the fish were equally distributed over 2 identical tanks (volume of 550 l) and kept with continuous freshwater flow (water temperature varying between 6.0 and 11 °C) in automated naturally simulated photoperiod. Fish were left to acclimatize for 2 weeks. After acclimatization one fish every day was attributed to either a control or an exposed group and exposed for 5 days to the pesticide mixture described in Table 5.4 or to tank water in static freshwater condition. At the end of the 5 days exposure each fish was removed from the experimental tank and transferred to a respirometer chamber filled with full strength seawater. Metabolic rate was determined by measuring oxygen consumption – MO_2 – as described in Chapter 2.

5.4.2. Results and discussion

Migratory behaviour of silver eels

Fourteen tagged eels from the Control Group (70%) and 13 eels from the Exposed Group (65%) were subsequently detected by at least one of the receivers located in the freshwater and estuary sections of the River Avon (Tables 5.6 and 5.7).

The migration of both groups of tagged eels in freshwater was predominantly nocturnal although two eels were recorded moving during the late afternoon. The majority of eels initiated their seaward migration 1-2 hr after sunset and migratory behaviour ceased 1-2 hr before sunrise. The mean time of day that the two groups were detected by the receivers in the freshwater section of the river are shown in Table 5.6. The mean migration speed ± S.E.M. from the release site to the estuary was not significantly different between the two groups of eels (Control: 0.202 ± 0.056 m s^{-1}; Exposed: 0.273 ± 0.065 m s^{-1}; T = 118.000, P = 0.406). The Control Group movement throughout the freshwater section of the River Avon was non-random with respect to the time of day with a significant mean time of movement during darkness. For example, in the middle section of the river (Receiver 3) the mean time that the eels were detected was 20:05. The length of mean vector (r) was 0.871 and the Rayleigh Test (p) was 0.00001 (Table 5.6). This indicates that the fish were moving with a directional component in relation to the time of day.

Table 5.6 The downstream movements of the two groups of tagged eels at each of the 5 receivers located in the freshwater section of the River Avon in relation to the time of day. The mean times that eels were recorded passing each site have been calculated from the mean vectors (Batschelet, 1981). The *r* values provide a measure of randomness of movement in respect to time calculated using the Rayleigh test. The value of n represent the total number of eel detected at each receiver.

	Receiver 1		Receiver 2		Receiver 3		Receiver 4		Receiver 5	
	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
Mean Time	18:51	Random movement	18:34	Random movement	20:05	19:24	20:05	19:21	20:22	19:19
r	0.782	0.374	0.935	0.451	0.871	0.714	0.856	0.784	0.867	0.794
p	= 0.018	= 0.451	< 0.001	= 0.382	< 0.0001	< 0.001	< 0.0001	= 0.002	< 0.0001	= 0.003
n	6	6	6	6	10	12	9	9	10	8

However, in comparison, the initial freshwater migration of the Exposed Group recorded at the two most upstream receivers was random with respect to the time of day (Table 5.6 –Receivers 1 and 2). This random movement with respect to time did not continue and by the time the eels were recorded in the middle section of the river the movement was non-random with a mean time of 19:24 (Table 5.6).

There was no significant difference in the time of movement between both groups of fish as they entered the estuary (Receiver 6). Movement was non-random with mean times of 20:42 (Control Group) and 20:21 (Exposed Group) (Figure 5.24). Movement of both groups through the estuary continued to be non-random with respect to time and occurred exclusively during the hours of darkness (Table 5.7). However, there were insufficient detections to carry out any statistical analyses with Receiver 10. Eels migrating passed Receiver 9 are considered to have moved out to the marine environment. The movement of both groups of eels through the estuary was predominantly on an ebbing tide (Figure 5.19) with both groups moving out to sea on the prevailing tide (Table 5.8). There were no differences in the time within the tidal cycle that each group of eels were detected at the estuary receivers (Receiver 6: $t = -0.610$, 19 d.f., $P = 0.549$: Receiver 7: $T = 95.000$, $P = 0.100$; Receiver 8: $T = 104.000$, $P = 0.270$; Receiver 9: $t = 1.132$, 15 d.f., $P = 0.276$). The residency of the eels within the estuary was relatively short and there was no significant difference between the Control and Exposed Groups in the times taken to move out into the marine environment. The mean residency times ± S.E were Control Group 17.6 ± 11.07 h and Exposed Group 9.2 ± 4.82 h ($T = 68.000$, $P = 0.736$).

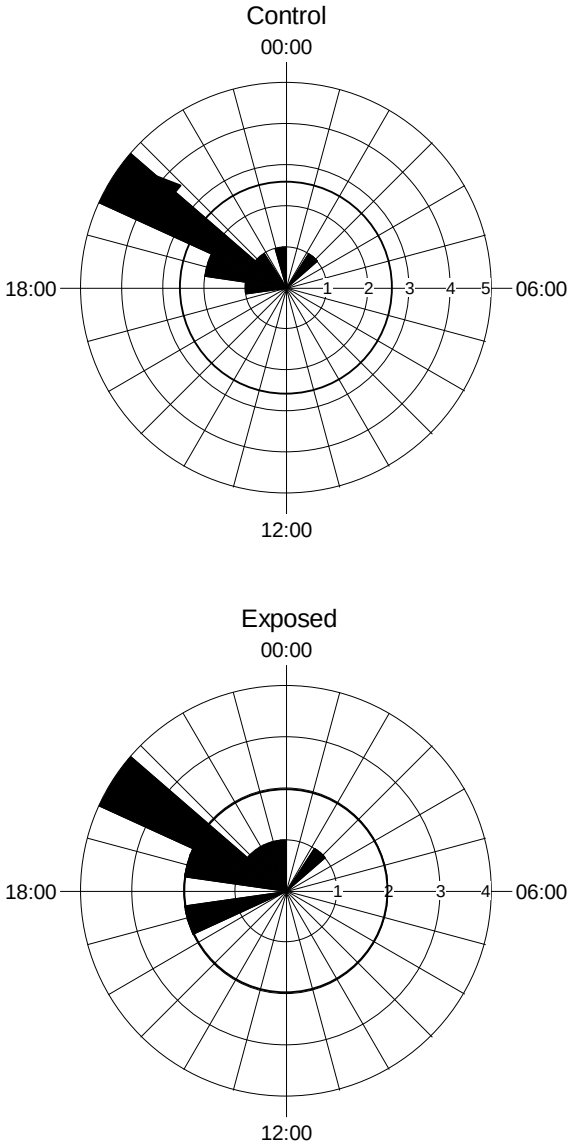


Figure 5.24 Histogram showing the time of day that the two groups of eels were detected migrating into the estuary of the River Avon (Receiver 6). The histogram provides an indication of the number of eels at each time. The arrow represents the mean time of detection calculated by vector analysis and its length represents the r value calculated using the Rayleigh test (Batschelet, 1981).

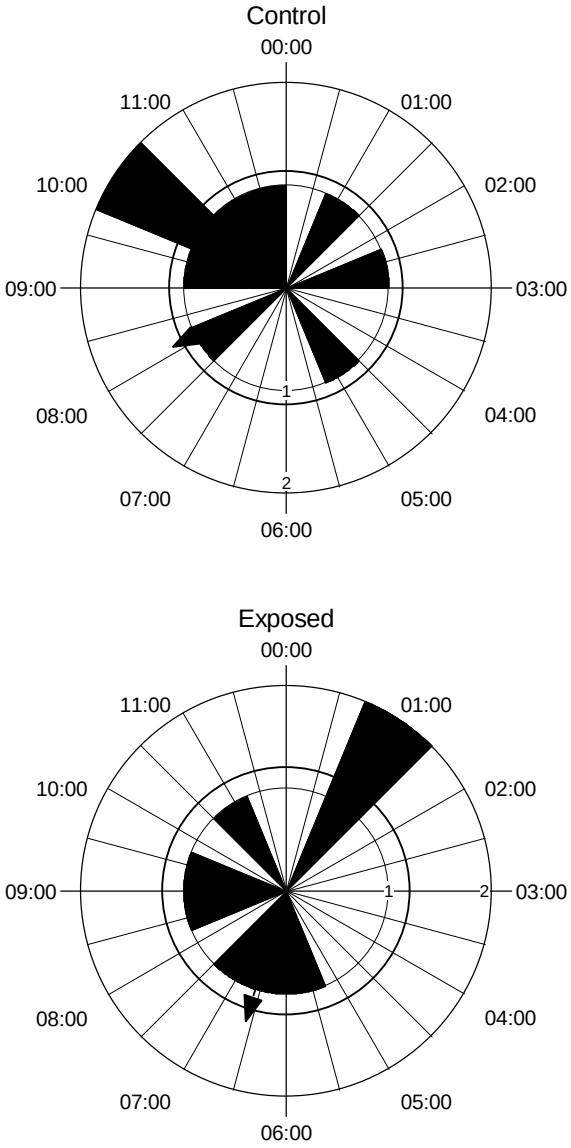


Figure 5.25 Histogram showing the movement of the two groups of eels leaving the River Avon estuary in relation to the tidal cycle (Receiver 9). The histogram provides an indication of the number of eels at each time. The arrow represents the mean time of detection calculated by vector analysis and its length represents the r value calculated using the Rayleigh test (Batschelet, 1981). The histogram shows the time in hours after the previous HW.

Table 5.7 The downstream movements of the two groups of tagged eels at each of the four receivers located in the estuary of the River Avon in relation to the time of day. The mean times that eels were recorded passing each site have been calculated from the mean vectors (Batschelet, 1981). The "r" values provide a measure of randomness of movement in respect to time calculated using the Rayleigh test. The value n is the total number of eels recorded at each receiver.

	Receiver 6		Receiver 7		Receiver 8		Receiver 9	
	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
Mean time	20:42	20:21	20:39	20:17	20:09	19:49	21:19	20:29
r	0.835	0.791	0.666	0.823	0.942	0.704	0.885	0.728
p	< 0.0001	< 0.0001	= 0.014	< 0.0001	< 0.0001	= 0.004	< 0.0001	= 0.01
n	11	12	9	11	9	10	10	8

Table 5.8 The downstream movements of the two groups of tagged eels at each of the four receivers located in the estuary of the River Avon in relation to the tidal cycle. The mean times that eels were recorded passing each site have been calculated from the mean vectors (Batschelet, 1981). The *r* values provide a measure of randomness of movement in respect to time calculated using the Rayleigh test. The value n is the total number of eels recorded at each of the receivers. All times are calculated from the previous 1st High Water measured at Christchurch Quay.

	Receiver 6		Receiver 7		Receiver 8		Receiver 9	
	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
Mean time	7 h 22min	7 h 39 min	9 h 09 min	7 h 07 min	8 h 29 min	7 h 25 min	8 h 05 min	6 h 34 min
r	0.72	0.876	0.76	0.654	0.731	0.74	0.622	0.662
p	= 0.002	< 0.0001	= 0.006	= 0.01	= 0.005	= 0.002	= 0.026	= 0.024
n	11	12	9	11	9	10	10	8

The results of the study suggest that exposure to a suite of contaminants may modify the migratory behaviour of emigrating silver eels at least during the initial freshwater stage. The movement of the exposed eels was random with respect to the time of day and not completely nocturnal as demonstrated by the control eels. However, the impact of the contaminants appeared to be short lived with the exposed eels establishing a more precise time of migration in the lower river and estuary with a significant mean time of movement during the early hours of darkness. The results are different from the previous study in the River Gudenaa, Denmark where there exposure to TBP did not modify behaviour or the timing of the migration in freshwater. The differences between the two studies may be the result of exposing the eels to a suite of contaminants rather than a single contaminant stressor.

The nocturnal emigration of the silver eels in the River Avon is similar to the results from other acoustic telemetry studies on eels carried out in rivers, estuaries and coastal waters (Aarestrup et al., 2010; Davidsen et al., 2011; Bultel et al., 2014). Aarestrup et al., (2010) showed that the migration in a fjord was predominantly nocturnal between 18:00 and 07:00 hr.

The migratory speed of both groups of eels were similar to those demonstrated by silver eels reported from other acoustic telemetry studies (Davidsen et al., 2011; Bultel et al., 2014) although more rapid than the eels reported by Aarestrup et al. (2010). The movement of the eels through the estuary of the River Avon was rapid and normally occurred within a single tidal cycle. Movement was predominantly on an ebbing tide which is the most energetically favourable method to move seawards and is similar to the migratory behaviour of other diadromous fish that have been studied in the River Avon (Moore et al. 1992; Moore & Potter, 1994). Movement was also nocturnal, and the rapid emigration on an ebbing tide during the hours of darkness could be envisaged as an anti-predator response similar to that reported for emigrating salmon smolts (Moore et al., 1995).

Respirometry and physiology of silver eels.

No mortality occurred during the exposure period or while the eels were in the respiromenter. Infestation with the swim bladder parasite *Anguillicola* was prevalent with only 20% of the eels being free from parasite and the rest of the fish having some

parasites in their swim bladder. The results of this part of the study show that there were no differences in either the Standard Metabolic Rate (SMR) or the Routine Metabolic Rate (RMR) of eels exposed to the mixture of pesticides when compared to the control group (Table 5.9). In addition, the results indicated that there were no negative effects of exposure to the mixture on eel morphological or physiological parameters. None of the measured parameters linked to osmoregulation (plasma ions, osmolarity and ATPase activity) indicated an effect of freshwater exposure to this mixture. The results are similar to the previous studies with fenitrothion and TBP.

Table 5.9 The effect of a pesticide mixture on various physiological and morphological parameters in silver eels whilst exposed in freshwater and then transferred in a respirometer chamber with full strength seawater for 24 hours. The data represents mean ± S.E.M. of 8 eels per group.

Morphological and physiological parameters	Control Group		Exposed Group		t-test
	mean	± sem	mean	± sem	Significance at > 0.05
weight (g)	180.72	29.6	161.4	29.68	0.65
length (cm)	43.69	2.31	42.65	2.37	0.76
Eye Index	5.87	0.58	6.98	0.63	0.21
fat content (%)	19.07	1.41	21.71	1.5	0.22
Condition Factor	0.2	0.007	0.2	0.008	0.44
Hepatosomatic index	1.39	0.1	1.4	0.07	0.94
Gonadosomatic index	0.54	0.25	0.47	0.21	0.83
gill ATPase (µmol Pi(mg hr)$^{-1}$)	7.62	1.12	5.44	1.06	0.18
kidney ATPase (µmol Pi(mg hr)$^{-1}$)	7.52	1.04	6.78	0.5	0.54
SMR (mg O_2 kg^{-1} hr^{-1})	16.2	1.48	20.05	2.83	0.26
RMR (mg O_2 kg^{-1} hr^{-1})	20.42	1.78	23.74	4.03	0.48
Osmolarity (mosm kg water^{-1})	390.87	7.96	386.87	11.85	0.78
Plasma chloride (mmol^{-1})	145.81	3.46	144.19	6.91	0.84
Plasma potassium (mM)	5.1	0.2	4.12	0.48	0.09

In conclusion, exposure of silver European eels to the pesticide mixture tested for a short period does appear to have an affect on their migratory behaviour only in the early stages of the freshwater migration but had no consequences on the migratory behaviour further down the river system or their physiological abilities to adapt to the marine environment. Further, this mixture does not appear to carry a metabolic cost to the eels at least in the short term.

Water samples from the experimental tanks were collected at the beginning, middle (only for exposed tanks) and end of experiment and sent to the National Laboratory Service for analysis. Results from the water analysis are presented in table 5.10 and indicate that exposed tanks had a lower concentration of each compound than the nominal concentration tested. This could be due to evaporation, absorption of the compounds to the tank sides and fittings or absorption from the fish.

Table 5.10 Actual concentration in µg l^{-1} of the pesticides studied in the experimental tanks.

Treatment		Control	Exposed	Exposed	Control	Exposed
Sample taken		19/12/2012	19/12/2012	26/12/2012	31/12/2012	31/12/2012
Analyte	expected					
Pendimethalin	0.8	<0.01	0.097	0.0204	<0.01	<0.01
Chlorpyrifos	0.05	<0.002	<0.002	<0.002	<0.002	<0.002
Chlorotoluron	0.5	<0.01	0.182	0.156	<0.01	0.133
Metaldehyde	0.2	0.058	0.187	0.221	0.055	0.23

5.5. Fluoxetine and metabolic cost

In January 2013 a group of 16 saltwater adapted PIT (Passive Integrated Transponder) tagged silver eels became available from a batch originally collected in the River Stour (Dorset) the previous year. As a result, a further study was undertaken to investigate the effect of short term exposure to a common pharmaceutical on the physiology and metabolic rate of eels within sea water. The compound chosen for the study was fluoxetine, which is routinely monitored in both river and estuarine environments.

Fluoxetine (Figure 5.26) is used as antidepressant (active ingredient of Prozac) and it acts as a selective serotonin reuptake inhibitor (SSRIs).

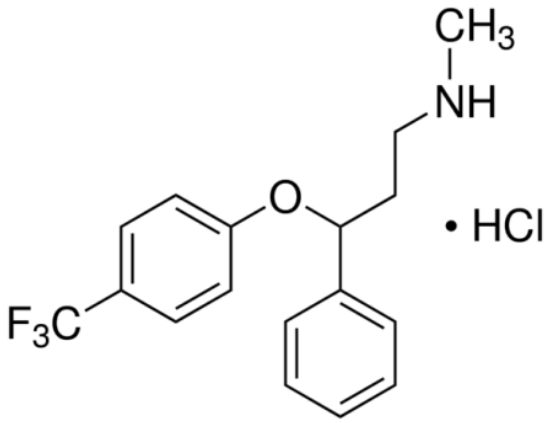


Figure 5.26 Flouxetine chemical structure.

It was one of the first SSRIs developed for clinical use as antidepressant and it is one of the most commonly prescribed (Paterson & Metcalfe, 2008). Several studies have detected environmentally relevant concentrations of fluoxetine ranging between 0.012 and 0.54µg l^{-1} (Weston et al., 2001; Kolpin et al., 2002; Metcalfe et al., 2003; Glassmeyer et al., 2005; Chen et al., 2006; Gagne et al., 2006). Few studies have been looking at the behavioural effects of fluoxetine in aquatic organisms. De Lange et al. (2006) investigated exposure to low concentrations (10–100 ng l^{-1}) of fluoxetine and found that it did significantly decreased activity in an amphipod. However, at higher concentrations (1µg l^{-1} to 1mg l^{-1}) no significant change in activity was observed. Henry & Black (2008) found that exposure to fluoxetine altered swimming behaviour in the mosquito fish, *Gambusia affinis*, which included changes to the normal position in the water column. Compared with the controls, the exposed fish appeared less responsive and lethargic (Henry & Black, 2008). Fluoxetine has also shown to affect

behavioural and biochemical responses in hybrid striped bass. Exposure to sub-lethal concentrations (35, 75 and 150 µg l^{-1}) of fluoxetine for 6 days reduced the species abilities to capture their prey resulting in reduced feeding and potentially ecological fitness (Gaworecki & Klaine, 2008). Additionally, after a 6-day non-exposure period the serotonin levels had still not recovered in all three treatments. In another study, Guler & Ford (2010) exposed a marine amphipod to concentrations of fluoxetine ranging from 0.01 to 10 µg l^{-1} and observed that amphipods exposed for 3 weeks to fluoxetine spent more time high up in the water column and in lighter areas than did the control animals. Standard ecotoxicity tests have found an LC_{50} for fathead minnow of 705 µg l^{-1} and of 2 mg l^{-1} for rainbow trout (Brooks et al, 2003).

5.5.1. Methods

Eels were maintained in one 700 l tank with a continuous seawater flow under an automated naturally simulated photoperiod. For the experiment one fish every day was attributed to either a control or an exposed group and exposed for 5 days to 0.1µg l^{-1} of fluoxetine in static saltwater or held in static clean seawater for control fish. The concentration of fluoxetine of 0.1µg l^{-1} chosen for this study was in line with concentrations measured in waters worldwide (Guler & Ford, 2010). Exposure was carried out in 550 l tanks supplied with constant aeration. At the end of the 5 days exposure each fish was removed from the experimental tank and transferred to a respirometer chamber filled with clean seawater. Each fish was left in the respirometer for 24 hours and the oxygen consumption was measured and logged during the 24 hr to allow the calculation of individual standard metabolic rate (SMR) and routine metabolic rate (RMR). After the 24 hours in the respirometer fish were removed and sampled for blood and tissue to allow physiological measurements. The parameters measured were both morphological (length, weight, organs weight, fat content) and physiological (plasma ions, osmolarity, gill and kidney Na/K ATPase).

5.5.2. Results and discussion

No mortalities occurred during the exposure period and while the fish were held in the respirometer. Assessment of the presence of the swim bladder parasite in all eels indicated an infestation rate of 71% with only 29% of the eels being free from *Anguillicola*. The results of the morphological and physiological data are summarized in table 5.11.

Table 5.11 Metabolic activity of silver eels exposed to fluoxetine in saltwater.

	Control Group		Exposed Group		t-test
	mean	± sem	mean	± sem	Significance at > 0.05
weight (g)	342.7	21.77	358.25	32.47	0.7
length (cm)	55.55	1.19	55.85	1.27	0.87
Condition Factor	0.2	0.005	0.2	0.007	0.77
Eye Index	6.05	0.62	7.31	0.58	0.16
fat %	17.47	1.4	16.92	1.75	0.81
Hepatosomatic index	1.2	0.07	1.08	0.04	0.18
Gonadosomatic index	0.96	0.18	1.06	0.16	0.68
gill ATPase (µmol Pi(mg hr)$^{-1}$)	13.44	1.44	11.26	1.57	0.32
kidney ATPase (µmol Pi(mg hr)$^{-1}$)	5.35	0.32	7.25	1.13	0.14
SMR (mg O_2 kg^{-1} hr^{-1})	11.23	0.94	10.35	0.51	0.43
RMR (mg O_2 kg^{-1} hr^{-1})	14.76	1.05	12.27	0.8	0.08
Plasma osmolarity (mosm kg water^{-1})	370.37	10.42	362.37	7.11	0.54
Plasma chloride (mmol^{-1})	151.62	2.63	146.88	2.84	0.24
Plasma potassium (mM)	6.39	0.65	6.9	0.44	0.53

There were no significant differences in either the SMR or the RMR of eels exposed to Fluoxetine when compared to the control group. In addition, the results of the experiment indicated that there were no negative effects of Fluoxetine on eel

morphological or physiological parameters. The results of the physiology study are similar to the previous studies on fenitrothion, a mixture of pesticides and TBP.

Fluoxetine is one of the most commonly detected pharmaceuticals in wastewater (Calisto & Esteves, 2009) and bioaccumulates in wild-caught fish, especially in brain, liver and muscle tissues (Paterson & Metcalfe 2008; Menningen et al 2010). Previous studies indicated that it is pharmacologically active exerting anorexigenic effects in fish species (Menningen et al., 2010) and inducing gamete liberation and alteration of endogenous levels of estradiol in the zebra mussel (Lazzara et al., 2012). Waterborne Fluoxetine has also been shown to regulate food intake and energy metabolism. Carp exposed for a period of 28 days to environmental levels of Fluoxetine showed a significant decrease in food intake and weight gain and the levels of circulating glucose levels (Mennigen et al., 2010). The authors examined the potential mechanisms and investigated gene expression of feeding neuropeptides in the neuroendocrine brain of goldfish as well as gene expression and enzymatic activity of glycolytic and gluconeogenetic enzymes in liver and muscle tissues. They were able to confirm changes in brain gene expression patterns in line with potential anorexigenic effects in the hypothalamus, with increased expression in corticotropin-releasing factor and decreased expression of neuropeptide Y. With respect to glucose metabolism, liver gene expression of the gluconeogenic enzyme fructose-1,6-bisphosphatase decreased and muscle hexokinase activity increased in fish exposed to Fluoxetine.

In the present study there appeared to be no effect on the glucose levels in the eel and although there was a lower RMR in the exposed group compared to the control group it was not significantly different. However, the eels in the present study were not exposed for as long a period as those in the work by Mennigen et al. (2010). It is possible that a longer exposure to the antidepressant, particularly during the freshwater stage may have more significant effects on feeding behaviour (not addressed in the present study) and subsequent, metabolism and bioenergetics of migrating eels. As stated previously any effects on the quality of emigrating silver eels has been suggested to reduce both migratory and reproductive success. Future studies investigating the role of pharmaceuticals on eels and the impact of changes to the metabolism in eels are warranted.

Chapter 6. General discussion

The major focus of this thesis was to investigate the potential impact of contaminants on what are considered to be sensitive life history stages of the European eel (*Anguilla anguilla* L.) and determine whether exposure to these substances that are present in freshwater, estuaries and coastal zones may be one of the reasons contributing to the decline of the species. In particular, the work examined how contaminants may modify or inhibit the physiological and behavioural processes that are necessary in allowing both juvenile and adult eels to move between the freshwater and marine environments. The results of this study suggests that exposure to environmental levels of the chosen freshwater contaminants, had very little effect on the osmoregulatory physiology although there was some evidence on the early migratory behaviour of the eels. A number of the morphological and physiological parameters commonly accepted as indicators of silvering in eels and saltwater adaptation (Durif et al., 2005) were not significantly modified by the majority of the contaminants that were investigated. As described earlier, this is very different to another well studied diadromous fish, the Atlantic salmon (*Salmo salar* L.) where exposure of juveniles to contaminants within freshwater did have a significant effect on the physiology of the fish particularly olfactory imprinting (Lower & Moore, 2007), saltwater adaptation and the ability of the fish to survive once they have migrated into the marine environment (Waring & Moore, 2004; Moore et al., 2003). However, the one similarity to the Atlantic salmon was in relation to the impact that the herbicide atrazine has on aspects of the physiology of saltwater adaptation. In this work atrazine was found to decrease the levels of kidney Na^+/K^+ATPase while in salmon smolts the reduction was observed in the gills (Waring & Moore, 2004). There has been a significant amount of research on atrazine (see Van der Kraak et al., 2014 for review) and it has been suggested that the toxic mechanism by which it operates on salmon smolts is through altering the ion flux activity within the fish gills (Waring & Moore, 2004). Whether, the pesticide would have a similar impact on silver eel gills or kidney and modify migratory behaviour and marine survival is not known. However, any reduction in one of the principal mechanisms relating to saltwater adaptation in the eel could have implication for both the short and long-term survival of the eel during its oceanic spawning

migration. In addition, studies on atrazine in combination with other contaminants have also shown effects on gill Na^+/K^+ATPase activity in salmon (Moore et al., 2003) and inhibition of acetylcholinesterase (Perez et al., 2013). While atrazine was studied in isolation, it is accepted that during its life cycle, the eel is exposed to a wide range of contaminants and although a single compound may not show a sub-lethal effect it is probable that there are additive and synergistic interactions among compounds, demonstrated in other fish species (Moore et al., 2003; Laetz et al., 2009 for review), that may have a significant impact on eel physiology and survival in the sea. This is supported by the results of the study on the mixture of contaminants on the run timing of the eels in the River Avon where there was a clear effect of mixtures of contaminants on the behaviour of migrating eels during the early part of the freshwater migration. Exposure to the "cocktail" of contaminants that are known to occur throughout the Avon catchment, as a result of run-off from agricultural practices, did modify the early migration pattern of the exposed eels in freshwater. The movement of the eels occurred randomly throughout the day and night and was not as directed as the control group. This is similar to other studies on migratory fish such as the Atlantic salmon where exposure to single and combinations of contaminants have significant effects on smolt migration and saltwater adaptation (Moore et al., 2007; 2008). Belpaire & Goemans (2007) reported that eels accumulated many of the contaminants that they were exposed during their residency in freshwater. The authors considered that exposure to contaminant cocktails may affect lipid metabolism and result in lower energy stores which in turn may be responsible for migration failure and/or impairment of reproduction. It is probable that exposure of eels to a suite of contaminants for extended periods during the freshwater phase of their life cycle may have a more significant long term impact on migratory behaviour in silver eels and subsequent reproduction. It is recommended that further studies are undertaken to look at the effects of contaminant "cocktails" focusing primarily on the energetics of migration, fecundity and spawning success on the eel.

The reason why the cocktail of contaminants modified the migratory behaviour of the eels but had little effect on the physiology and morphology is not clear. However, one possible explanation is that the contaminants had an effect on other physiological processes involving other compounds such as hormones that weren't monitored or measured in the migrating eels during the present research. For instance, the thyroid

hormones, triiodothyronine (T_3) and thyroxine (T_4) are involved in the migration of diadromous fish. Thyroid hormones play a role in the parr-smolt transformation in salmonids (Hoar, 1988; Boeuf, 1994) and have also been implicated in initiating the upstream and downstream movements of juvenile and adult salmonids (Youngson & Webb, 1993; Iwata, 1995; Hutchinson & Iwata, 1998). In addition, thyroid hormones have been considered to control the upstream migration of glass eels (Castonguay et al., 1990, Edeline et al., 2005a), although their role in initiating and controlling silver eel migration is not fully understood. Aroua et al. (2005) demonstrated a modest increase in thyroid hormones between the yellow and silver eel stages while van Ginneken et al. (2007b) observed no link between the circulating thyroid levels and silvering. However there is no information on the potential link between thyroid hormones and migratory behaviour. It is possible that the contaminant cocktail studied may have modified the thyroid levels in the eels which in turn disrupted the nocturnal migration of the eels in freshwater.

However, this was not case with one of the contaminants studied during the present research, the plasticizer and flame retardant tributyl phosphate (TBP). Although the role of TBP in modifying thyroid levels in fish is unknown, other flame retardants are known to target thyroid and reproductive systems in fish. There is evidence that one group of chemicals, polybrominated diphenyl ethers (PBDEs), disrupt the hypothalamic-pituitary-thyroid axis, thyroid hormones transport and metabolism, thyroid receptors and thyroid follicle histology (see Yu et al., 2105 for review). These chemicals also have a significant impact on disruption on steroid hormone production, expression of genes involved in steroidogenesis, and gonadal development. In this thesis, although the thyroid system was not studied, experiments to determine the impact of contaminants on thyroid hormones could have provided a useful insight into the role of these compounds in controlling migration and explain potential toxic mechanisms to the migratory behaviour of the eel. Further, the eels may be susceptible to flame retardants which may have a significant impact on the population. It is recommended that the impact of contaminants, such as PBDE are studied particularly on the interaction between thyroid and reproductive systems in fish and the relationships between reproductive toxicity and thyroid system disruption after PBDEs exposure.

The lack of significant effects on the aspects of the osmoregulatory physiology of the eels investigated in the present work, compared to studies on other diadromous fish, may reflect the short duration of exposure that was undertaken during the experiments. All the studies examined the exposure of the silver eels immediately prior to the transition from the fresh to the marine environment. However, eels are normally present in freshwater for periods in excess of 5 years and so long term exposure may be an additional issue in relation to the successful transition from fresh to salt water. However, it was not possible to expose eels for durations that represent the life cycle of the eel in freshwater. The eels sampled in the present study were also collected relatively low down the various river systems and so there is no evidence of their previous distribution within freshwater. Therefore, the pre-exposure of individuals to contaminants prior to the laboratory and field-based studies is not known. Adaptation and acclimation to the studied contaminants may therefore have played a role in the non-response of the experimental eels. It is recommended that further research should focus on long term exposure of eels to contaminants. However, it is accepted that such studies are logistically difficult given the residency period of eels in freshwater which may extend to many years. An alternative approach is to study adult eels from environments with very different quantified contaminant levels to try to understand the long term implications of pollution.

In the majority of the laboratory experimental studies the design incorporated the use of duplicate tanks per treatment. Although in acute toxicity testing the numbers of tanks per treatment is normally one per concentration (OECD, 1992), when examining more sublethal effects of environmental concentrations of contaminants it is recognised that the more replicates per treatment the more sensitive will be the results in indicating more subtle changes in the fish. However, duplicates were chosen in this study due to the influence of the the principles of the 3Rs which are implicit in UK law under the Animals (Scientific Procedures) Act (1986). The three Rs are the guiding principles underpinning the humane use of animals in scientific research and stand for replace, reduce and refine. In this thesis the focus was on reducing the number of experimental animals particularly given that the work was relying on wild caught fish of a species that was below its safe biological level as a population.

The eel is known to have an acute sense of smell and in common with other teleosts that exist in waters with reduced visibility or that forage during the hours of darkness, the olfactory system plays a major role in prey detection and feeding behaviour. The studied contaminant did not reduce or inhibit the olfactory ability of the yellow eels to detect an amino acid commonly released by invertebrates and probably used as a cue in prey detection and feeding. Further, direct exposure to contaminants within the substrate and in the food of experimental animals did not have any significant effect on a range of physiological processes, feeding behaviour or growth. However, the present work only focused on the short term exposure to contaminants, the impact of exposure over a period of months on olfactory function, prey detection, feeding behaviour and growth may be significantly greater and have a deleterious effect on individuals within the population. Recently, it has been demonstrated that a reduction in lipid levels and condition factor has occurred in yellow eels which coincides with the decline in the population (Belpaire, 2008). The authors suggest that contaminants may be involved but do not propose any possible toxic mechanism. However, any factor that has a direct effect on the sense of smell in fish, such as pesticides (Moore & Waring, 1998), may result in a reduced feeding success in eels and explain the reduced fat content. Reduced energy reserves in the eel necessary for the long spawning migration may also reduce gonad development and spawning success. Longer term studies on olfactory function and contaminant exposure are recommended in order to understand any potential toxic relationship between pollution and the sense of smell in the eel. The olfactory experiments did produce one very novel finding. The work examined the ability of the eel to detect the compound 20-Hydroxyecdysone (ecdysone) a naturally occurring ecdysteroid hormone which controls the ecdysis (moulting) and metamorphosis of arthropods. It is one of the most common moulting hormones in insects and crustacean. The rationale behind the study was to investigate whether fish are able to detect this compound in the water at the time when many of their common prey items are moulting and may be more susceptible to detection and predation. Ecdysone was detected by the olfactory system of the eel and this is the first time a teleost has been shown to have a direct olfactory response to an ecdysteroid hormone. Previously, fish were known to only detect a range of teleost reproductive hormones that controlled and synchronised much of the spawning physiology and behaviour between the males and females (Stacey, 2003). Ecdysone, may be one of the most common odorants used by teleosts to detect their

prey and within the marine environment may explain why the soft shelled crabs undergoing moulting and growth are preyed upon preferentially by fish such as the European bass.

Although the research indicated that exposure to key contaminants had no effect on the short term migration of silver eels, it is possible that exposure to contaminants could have a greater effect on the ability of silver eels to undertake their marine migration and their spawning viability. Bioaccumulation of toxins is a concern and many studies have indicated that eels are particularly prone to the uptake of lipophilic contaminants such as polychlorinated biphenyls, organo-chlorine pesticides and brominated flame retardants which are stored in the fat tissue (Geeraerts & Belpaire, 2010). In the yellow stage, eels are sub-adults and do not reproduce. Unlike iteroparous fish, eel's body-burden is not affected by the reproductive cycle as there is no loss of contaminants occuring with reproduction and the associated changes in lipid metabolism (Geeraerts & Belpaire, 2010). The contaminants accumulated throughout the eel growing phase would become available during the metabolism of the fat reserves necessary for migration and could result in reduced migratory success in the marine environment. It has previously been shown by Belpaire (2008), that there is also a link between the declining eel population and a decrease, on average, of one third of the fat content in yellow eels together with a decline in relative condition factor. The authors estimated the reproductive potential of eels from various latitudes over Europe, on the basis of the somatic energy reserves, assuming that fat levels in yellow eel are indicative of those in silver eels. Only large individuals, females as well as males, with high lipid content seem to be able to contribute to the spawning stock. The authors concluded that the decrease in fat content in yellow eels may be a key element in the stock decline and raises serious concerns about the chances of the stock to recover. The combination, of high contaminant levels in fat and reduced levels of lipids in the migratory eels could operate in an additive or synergistic manner to reduce survival during the marine phase even though the individuals have been successful during the short term transition from rivers to the coast. Research, particularly, on the impacts of contaminants on eel reproductive biology, is required but until it is possible to close the eel life cycle and successfully produce viable individuals within hatchery facilities, the direct effects of the freshwater and marine environment in regulating the population will remain conjecture. In a similar way the logistics and difficulties of

studying the behaviour and energetics of actively migrating silver eels will reduce our abilities to understand the factors operating in the sea that also regulate this phase in the life cycle of the eel.

The thesis has produced some very detailed information on the temporal and spatial patterns of eel migration within freshwater and estuaries that will support their management and conservation. The work demonstrated that the movement of eels in freshwater was predominantly nocturnal; migration was generally initiated 1-2 hours after sunset and ceased 1-2 hours before dawn. This behaviour was extended during the transition through the estuary and entry into the marine environment where fish moved seaward, predominantly on an ebbing tide. This type of information is important in mitigating the potential effects of freshwater and coastal hydropower schemes on silver eels. One major concern is that turbines associated with in-river, estuarine and coastal schemes cause significant mortalities and delays to migrating eels (Winter et al., 2006; Piper et al., 2012, 2013; Buysse et al., 2014). Due to their elongated shape, eels are more susceptible to damage during the downstream passage through a turbine than other diadromous fish (Buysse et al., 2014). In the River Frome in England, recent telemetry studies have indicated that despite screening, eels do move through an Archimedes Screw turbine associated with an in-river hydropower scheme (personal observation) and that this occurs principally during the hours of darkness. Modifying the operation of the hydropower scheme and generating power only during the daylight hours when the fish are migrating would significantly reduce the impact on the eels. Similar advice on the operation of prospective new developments such as the Swansea Bay Tidal Lagoon project would assist in reducing damage to eels as the result of their passage through the turbines generating the electricity. In particular, reducing the operation of the turbines during an ebbing tide would also reduce the numbers of eels entrained in the turbines.

In a similar way to the silver eels, there was no evidence that exposure to the marine contaminants investigated had an effect on the ability of glass eels and elvers to physiologically adapt to and successfully survive the transition from the marine to the freshwater environment. Although, the short term exposure of glass eels to contaminant did not affect the success of the transition from the marine to freshwater environment, the present study did indicate that exposure to certain metals within sea water produced significant genetic damage in the glass eels as measured by the Comet

Assay. Damage at the molecular level in many fish can cause serious problems but it is not clear to what extent the genetic damage is carried through to the whole animal or what the effects might be at the population level. In addition, the Comet Assay technique does not differentiate between transient and permanent damages to the DNA, preventing the ability to draw conclusions on the long term effects of chemical exposure. The reduced DNA damage measured by the Comet Assay in the experiment with eel elvers exposed for five weeks as opposed to the high damage measured in glass eels exposed for two weeks could suggest that this damage was transient and the DNA repair mechanisms present in the cells could prevent long term deleterious effects. Although, eels are known to bioaccumulate a number of toxins as a result of their high fat content (Geeraerts & Belpaire, 2010), the present research also indicated no physiological or behavioural effects on juvenile eels as a result of exposure to contaminated sediment or ingested contaminated prey. However, once again the Comet assay carried out on the elvers did indicate that exposure to metals in the sediment does cause some genetic damage although how this is can be assessed at the whole animal and population level is not clear.

The research undertaken for this thesis specifically examined the potential impact of environmental levels of contaminants on the various life history stages of the eel in order to understand the mechanisms reducing the eel population. In general, the contaminants studied had very little effect on the eels at these concentrations, but it is accepted that at higher doses changes to the behaviour and physiology of the eels are possible. Positive controls and dose response corves for each of the contaminants were not undertaken during the study but would have been useful in indicating the potential toxicity of the targeted contaminants.

The principal driver for undertaking this thesis was the significant decline in the population of European eel and the need to identify factors that may be responsible, in order to better implement the Eel Recovery Plans brought in by the European Commission in 2007. At that time the glass eel recruitment had decreased dramatically across Europe and the eel stock was considered to be outside safe biological limits (ICES, 2006). In 2009, the European Eel was also listed in CITES Appendix II and in Annex B to Council Regulation (EC) No 338/97 and it was concluded that the situation of the stock was too critical to allow international trade without detriment to the survival of the species. Exports from and imports into the EU of the eel were therefore

suspended. Unfortunately, the most recent assessments of the eel population remain the same. In 2013 (ICES, 2013), based on the information (stock indicators) provided by EU Member States, it was concluded that the stock, at least in the reporting countries, was not within sustainable limits conforming to the Eel Regulation and ICES policies. The biomass of escaping silver eel was estimated to still be well below the target of 40% set in the EU Eel Regulation. The European eel still remains on the danger list.

However, during the period that this thesis was being undertaken, further work has been published that suggest other factors that may be involved in the recent eel decline (Baltazar-Soares et al., 2014). The authors suggest that regional atmospherically driven ocean current variations in the Sargasso Sea were the major driver of the onset of the sharp decline in eel recruitment in the beginning of the 1980s. The oceanic current simulations combined with genotyping of natural coastal eel populations suggest that unexpected evidence of coastal genetic differentiation is consistent with cryptic female philopatric behaviour within the Sargasso Sea. Such results demonstrate the key constraint of the variable oceanic environment on the European eel population (Baltazar-Soares et al., 2014). In addition to the changes in oceanic current patterns there is strong evidence for the acidification of the Atlantic Ocean (Caldeira & Wickett, 2003; 2005). Ocean acidification, caused by increasing atmospheric concentrations of CO_2 is one of the most critical anthropogenic threats to marine life (Frommel et al., 2012). Changes in seawater carbonate chemistry have the potential to disturb calcification, acid–base regulation, blood circulation and respiration, as well as the nervous system of marine organisms, leading to long-term effects such as reduced growth rates and reproduction. In teleost fishes, early life-history stages are particularly vulnerable as they lack specialized internal pH regulatory mechanism. In juvenile cod for instance exposure to CO_2 resulted in severe to lethal tissue damage in many internal organs, with the degree of damage increasing with CO_2 concentration (Frommel et al., 2012). The authors concluded that as larval survival in many fish species is the bottleneck to recruitment, ocean acidification has the potential to act as an additional source of natural mortality, affecting populations of already exploited fish stocks. The leptocephali of the eel would also be very vulnerable and susceptible to acidification during their migration back to Europe. Whether acidification is an additional reason for the low recruitment of glass eels is

not known but laboratory studies on the effects of CO_2 exposure on the early life stages of eels is recommended.

The invasive parasite *Anguillicola crassus* found in the eel swim bladder is also considered to be a significant factor contributing to the population decline (Emde et al. 2014). *A. crassus* is a parasite of Japanese eel and it was originally endemic to East Asia (Kirk, 2003). It was probably introduced with eels imported from Taiwan and has successfully invaded Europe and the European eel. The first records of *A. crassus* in Europe date back to 1982 in North-West Germany and can now be found in the eel stocks throughout most of Europe. The first reports of the parasite in the UK are from various rivers in 1987 (Kirk, 2003). Previous studies have indicated that the European eel is more susceptible to *A. crassus* than the Japanese eel. The Japanese eel has co-evolved with the parasite and so its immune system is probably more effective against the larval stage. The Japanese eel is also capable of eliminating the parasite after vaccination or under high infection pressure, but this has not yet been observed in European eels (Emde et al. 2014).

Infestations with *A. crassus* lead to significant impairment of the swim bladder function and reduced swimming performance. This can have a major impact on the ability of eels to complete the 5000 km spawning migration during which they undertake daily vertical migrations between depths of 200 and 1000 m.
The eel, is the final host for the parasite *A. crassus,* where the adults are localized exclusively in the swim bladder, where they feed on blood and reproduces. Embryonated eggs as well as hatched larvae, leave the eel via the *ductus pneumaticus* of the swim bladder and the intestinal tract. *A. crassus* uses different invertebrates (especially planktonic crustaceans) as obligate first intermediate hosts for the development of its third-stage larvae, which is infective for the eel (e.g. so far, 23 different crustacean species, mostly copepods could be identified as first intermediate hosts). Additionally, 50 paratenic hosts such as several insect and amphibian species as well as at least 37 fish species can be incorporated in the life cycle. The paratenic hosts accumulate large numbers of parasites, either freely in the body cavity, or in and on organs such as the gonads, intestinal wall and swim bladder, and thus, bridge the trophic levels between larger piscivorous eels and copepods. Smaller eels get infected predominantly by feeding on parasitized crustacean intermediate hosts, whereas larger

eels, preying mostly on fish, ingest infective larvae with paratenic fish hosts (Emde et al., 2014). Most eels used in the experiments presented in this thesis had various degrees of parasite infetsation. The limited number of fish sampled for this work and the number of other variables between the various fish batches do not allow to calculate an overall infection rate but further studies investigating the occurrence and quantity of parasite in eels from different environments (e.g. different salinities, temperature, population density) would provide useful information on the potential link and connsequences between *A. crassus* infestation and population decline and moreover valuable information in support of eel restoration plans.

In addition to the many contaminants already studied, both in this thesis and in the published literature, there are always new and novel chemicals being developed and which find their way into the aquatic environment. For instance, there are a wide-range of emerging organic groundwater contaminants (EGCs) which are beginning to be monitored in the UK. These include nano-materials, pharmaceuticals, industrial compounds, personal care products, fragrances, water treatment by products, flame surfactants as well as caffeine and nicotine. Although their detection within the aquatic environment is more recent than the decline in the eel population they are also of concern as their toxic mechanisms are poorly understood and the sub-lethal effects on the physiology and behaviour of the eel are unknown. Therefore, it is recommended that future targeted studies investigate the role of these EGCs on eels and their impact on the biology of eel throughout its complex life-cycle.

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Appendix 1. Pesticides in Hampshire

Estimated annual use of pesticides in Hampshire - kg active substance applied per month (estimate for years 2004/2005, data provided by Environment Agency).

Active substance	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Annual
2,4-D	.	.	.	.	30.38	308.94	.	.	.	156.42	.	.	495.74
2,4-DB	.	.	.	.	4,101.55	.	.	.	.	.	.	.	4,101.55
2-chloroethylphosphonic acid	.	.	.	.	1,259.23	165.79	.	.	.	.	.	.	1,425.02
Abamectin	.	.	< 0.01	.	.	.	< 0.01	.	.	.	.	.	0.55
Alpha-cypermethrin	.	.	2.28	29.34	72.49	15.44	.	.	11.3	4.4	45.82	.	181.07
Amidosulfuron	.	.	12.56	51.49	3.5	.	.	.	.	.	.	.	67.54
Amitraz	.	.	.	.	.	.	13.03	13.03	.	.	.	.	26.06
Asulam	.	.	.	.	2.73	.	.	.	.	.	.	.	2.73
Atrazine	.	8.37	18.26	1,973.61	2,849.37	1,189.44	.	.	.	.	.	.	6,039.06
Azoxystrobin	.	.	.	352.85	1,461.11	1,838.74	159.91	.	.	.	< 0.01	.	3,812.62
Bacillus thuringiensis var. kurstaki	.	.	.	.	< 0.01	.	.	< 0.01	< 0.01	.	.	.	0.97
Benazolin	.	.	2.5	.	.	.	.	.	.	.	.	80.16	82.66
Benodanil	.	.	.	.	.	.	.	.	< 0.01	.	.	.	0.21
Benomyl	.	.	.	.	.	.	5.04	.	.	.	.	.	5.04
Bentazone	.	.	.	154.12	557.81	159.16	.	.	.	.	.	.	871.08
Beta-cyfluthrin	.	.	62.35	37.78	.	.	.	102.58	1.61	.	.	.	204.33
Bifenthrin	< 0.01	< 0.01	< 0.01	< 0.01	.	.	< 0.01	.	.	5.44	4.59	4.24	16.14
Bitertanol	.	.	.	.	.	.	.	79.46	1,998.64	866.13	52.51	1.83	2,998.58
Boscalid	.	.	.	470	1,263.88	.	.	.	.	.	.	.	1,733.87
Bromacil	6.23	3.05	.	.	.	.	.	.	.	.	.	.	9.29

Active substance	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Annual
Bromoxynil	.	.	769.23	369.86	1,726.05	689.1	.	.	.	.	.	.	3,554.23
Bupirimate	< 0.01	1.85	< 0.01	< 0.01	4.77	34.41	5.67	32.38	< 0.01	< 0.01	.	.	79.6
Buprofezin	.	.	.	< 0.01	.	.	.	.	.	.	.	.	0.72
Captan	.	.	.	153.37	13.34	233.7	.	.	.	.	.	.	400.4
Carbendazim	< 0.01	384.79	91.98	353.01	1,191.45	473.7	< 0.01	.	< 0.01	.	489.67	.	2,985.47
Carbetamide	.	.	823.53	.	.	.	.	.	.	.	1,682.09	.	2,505.63
Carbosulfan	.	.	.	.	.	.	.	8.04	.	.	.	.	8.04
Carboxin	.	.	.	.	.	.	.	.	217.29	178.38	.	.	395.67
Carfentrazone-ethyl	.	.	< 0.01	.	.	.	.	.	.	1.79	11.26	29.85	43.1
Chlormequat	.	.	11,397.71	45,924.01	11,170.41	.	.	.	.	.	.	.	68,492.11
Chlormequat chloride	.	.	.	.	187.3	.	.	.	.	.	.	.	187.3
Chlorothalonil	8.86	23.35	3,342.86	10,446.50	15,748.82	8,614.28	121.06	19.16	24.64	16.38	8.41	29.17	38,403.50
Chlorotoluron	.	.	.	.	.	.	.	.	.	4,898.54	8,948.17	.	13,846.71
Chlorpropham	.	.	.	.	.	.	.	.	.	.	.	.	1,148.53
Chlorpyrifos	.	.	60.8	5.67	108.34	4,283.55	350.39	127.99	.	2.05	16.07	.	4,954.84
Chlorthal-dimethyl	.	.	.	306.95	22.31	.	.	.	.	.	.	.	329.26
Clodinafop-propargyl	.	29.69	.	42.18	97.14	28.04	.	.	.	5.77	1.76	63.55	268.13
Clofentezine	.	.	.	1.34	.	< 0.01	< 0.01	.	.	.	.	.	2.07
Clomazone	.	.	171.49	.	.	.	.	127.29	107.77	.	.	.	406.55
Clopyralid	.	.	49.11	71.38	41.57	< 0.01	.	.	.	.	.	14.87	177.2
Copper oxychloride	.	.	101.74	.	4.48	.	.	< 0.01	17.92	178.18	128.79	15.07	447.06
Cupric ammonium carbonate	.	.	.	.	.	< 0.01	.	< 0.01	.	.	.	.	0.21
Cyanazine	142.84	160.81	444.67	317.64	111.1	37.86	.	.	.	.	.	401.17	1,616.09
Cycloxydim	.	.	33.14	43.56	105.14	.	.	.	25.21	.	72.22	324.55	603.82
Cymoxanil	.	.	30.47	.	.	.	.	.	.	.	.	.	30.47
Cypermethrin	< 0.01	23.5	2.19	49.84	245.39	174.75	1.42	15.91	180.32	52.2	557.18	294.19	1,596.96
Cyproconazole	.	.	127.32	211.65	97.5	243.7	.	.	.	.	.	.	680.16
Cyprodinil	.	.	.	3,628.44	1,794.09	834.35	.	.	.	.	.	.	6,256.88
Daminozide	.	.	.	.	.	.	.	.	.	.	.	.	166.65
Deltamethrin	.	.	< 0.01	9.31	15.38	1.42	.	< 0.01	< 0.01	2.83	3.04	8.51	40.77
Dicamba	.	.	.	.	4.14	11.29	< 0.01	.	.	12.04	< 0.01	< 0.01	28.15
Dichlobenil	2.75	129.98	58.74	.	.	.	.	.	.	.	.	.	191.48

Active substance	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Annual
Dichlofluanid	.	.	24.52	188.95	366.86	152.69	34.49	.	.	.	.	.	767.51
Dichlorophen	.	.	315.73	.	.	.	.	.	.	.	.	.	315.73
Dichlorvos	.	.	.	.	.	.	.	.	.	.	.	.	50.68
Diclofop-methyl	.	10.61	.	11.48	258.68	.	.	.	.	93.9	.	22.26	396.92
Dicofol	.	.	.	.	.	.	1.78	.	.	.	.	.	1.78
Difenoconazole	.	104.36	.	.	.	.	.	.	.	.	185.9	.	290.26
Diflubenzuron	.	.	.	.	.	15.47	.	.	.	.	.	.	15.47
Diflufenican	.	15.59	120.47	13.99	.	.	.	.	.	50.4	292.84	254.82	748.12
Dinocap	.	.	.	.	.	.	4.3	.	.	.	.	.	4.3
Diquat	1.21	39.32	1.92	5.3	296.61	34.68	1.21	154.04	1.21	.	.	.	535.49
Dithianon	.	.	10.32	264.51	17.54	9.31	.	.	.	.	.	.	301.68
Diuron	.	57.46	114.36	.	.	.	.	.	.	.	.	.	171.82
Endosulfan	.	.	.	.	< 0.01	< 0.01	.	.	.	.	.	.	1.65
Epoxiconazole	.	.	555.99	2,300.92	4,000.80	534.78	.	.	.	.	.	3.75	7,396.24
Esfenvalerate	.	< 0.01	.	.	.	6.93	.	.	.	8.03	25.41	.	40.95
Fatty acids	.	3.64	.	27.34	26.61	20.06	23.7	9.74	.	3.64	.	.	114.73
Fenarimol	.	.	.	< 0.01	2.19	< 0.01	5.17	< 0.01	.	.	.	.	9.8
Fenbuconazole	.	.	.	.	.	< 0.01	.	.	.	.	.	.	0.84
Fenbutatin oxide	.	.	.	.	.	.	.	52.26	7.47	.	.	.	67.19
Fenhexamid	.	.	.	.	22.49	27.4	8.23	3.83	.	.	.	.	61.96
Fenitrothion	.	.	.	6.57	.	.	.	.	.	.	.	.	6.57
Fenoxaprop-P-ethyl	.	< 0.01	10.33	2	20.7	.	.	.	.	12.33	.	1.78	47.99
Fenpropathrin	.	.	1.91	1	1	.	.	.	.	.	.	.	3.91
Fenpropidin	.	.	.	.	281.5	223.66	.	.	.	.	.	.	505.16
Fenpropimorph	.	.	511.28	3,100.21	7,952.50	646.82	.	2.74	10.51	.	.	.	12,224.05
Fenpyroximate	.	.	< 0.01	< 0.01	.	.	< 0.01	.	.	.	.	.	0.67
Fenuron	.	.	.	.	.	.	.	.	.	.	.	.	215.52
Flamprop-M-isopropyl	.	.	.	.	195.4	.	.	.	.	.	.	.	195.4
Florasulam	.	.	27	37.27	8.53	2.97	.	.	.	.	.	.	75.78
Fluazifop-P-butyl	.	201.39	90.25	73.44	34.4	.	.	.	.	.	314.04	.	713.52
Fludioxonil	.	.	15.23	< 0.01	< 0.01	.	.	.	12.99	2.97	1	.	33.13
Flufenacet	.	23.88	.	.	.	.	.	.	493.21	265.06	184.98	.	967.12
Flupyrsulfuron-methyl	.	12.86	< 0.01	.	.	.	.	.	< 0.01	11.81	34.97	22.28	82.56

Active substance	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Annual
Fluquinconazole	.	.	.	.	98.36	.	.	.	.	.	.	.	98.36
Fluroxypyr	.	.	< 0.01	1,862.57	2,319.55	334.8	< 0.01	.	.	.	.	4.81	4,523.37
Flurtamone	.	21.17	.	.	.	.	.	.	.	73	149	60.92	304.1
Flusilazole	.	8.31	459.41	101.1	820.14	44.79	.	.	.	34.73	1,136.12	211.03	2,815.63
Fosetyl-aluminium	29.33	20.99	.	382.44	366.99	385	356.56	356.46	153.99	23.54	20.03	39.4	2,134.68
Fuberidazole	.	.	.	.	.	.	.	4.88	141.11	113.32	3.22	< 0.01	262.64
Furalaxyl	.	.	.	< 0.01	.	.	.	.	.	.	.	.	0.13
Gibberellins	.	.	.	.	1.11	< 0.01	.	.	.	.	.	.	1.57
Glufosinate-ammonium	1.75	13.99	14.01	.	1.59	1.95	4.21	2.67	.	.	.	.	40.17
Glyphosate	907.67	3,437.05	878.76	6,428.04	2,051.91	297.18	22,892.47	10,184.84	20,897.36	5,257.11	3,105.57	269.33	76,607.31
Guazatine	.	.	.	.	.	.	.	.	.	38.06	.	.	38.06
Heptenophos	.	< 0.01	.	.	.	.	.	.	.	.	.	.	0.94
Imazaquin	.	.	5.97	2.14	< 0.01	.	.	.	.	.	.	.	8.26
Imidacloprid	.	.	63.75	122.81	< 0.01	.	.	131.21	1,037.11	841.34	33.52	.	2,229.87
Iodosulfuron-methyl-sodium	.	< 0.01	3.33	< 0.01	.	.	.	.	.	.	< 0.01	5.27	10.32
Ioxynil	.	.	527.91	334.36	1,206.23	.	.	.	.	.	.	.	2,068.49
Iprodione	4.25	< 0.01	.	24.37	1,442.80	.	543.31	136.82	4.01	4.04	2.88	1.87	2,164.93
Isoproturon	25.15	429.24	10,064.07	3,607.06	.	.	.	.	1,174.33	4,852.54	31,200.56	14,010.11	65,363.06
Isoxaben	23.62	17.4	44.73	49.15	27.71	7.32	3.06	< 0.01	7.32	59.07	.	< 0.01	239.37
Kresoxim-methyl	.	.	426.06	1,168.65	1,514.41	113.05	.	.	.	.	.	.	3,222.17
Lambda-cyhalothrin	.	.	< 0.01	9.81	34.35	58.18	2.74	< 0.01	9.22	3.81	39.24	.	157.64
Lenacil	.	.	.	.	.	.	.	.	.	.	.	.	225.63
Linuron	.	.	< 0.01	.	561.58	.	.	.	184.63	.	.	.	746.4
Malathion	.	.	.	.	.	.	.	.	.	.	.	.	35.87
Mancozeb	.	2.51	41.37	41.89	.	1,064.34	517.14	.	19.34	6.37	.	.	1,692.94
MCPA	.	.	149.21	.	664.89	1.38	3.29	1,106.28	183.7	.	3.14	3.03	2,114.94
MCPB	.	.	.	.	389.52	159.16	.	.	.	.	.	.	548.68
Mecoprop-P	.	.	1,799.03	389.96	3,857.05	80.14	< 0.01	730.99	.	571.25	2,211.33	843.7	10,484.00
Mepanipyrim	.	.	.	.	< 0.01	.	1.18	< 0.01	.	.	.	.	1.39
Mepiquat	.	.	.	.	500.22	326.16	.	.	.	.	.	.	826.38
Mepiquat chloride	.	.	.	.	61.06	.	.	.	.	.	.	.	61.06
Mesosulfuron-methyl	.	2.98	13.89	2.24	.	.	.	.	.	.	2.69	26.36	48.16

Active substance	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Annual
Mesotrione	.	.	.	.	6.78	28.89	.	.	.	.	.	.	35.67
Metalaxyl	.	.	.	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	.	.	.	.	3.82
Metalaxyl-M	.	< 0.01	67.27	< 0.01	1.83	1.83	1.24	.	.	9.39	.	.	82.28
Metaldehyde	4.77	.	.	3.59	3.66	115.45	.	911.14	1,277.81	2,214.85	309.43	4.15	4,844.84
Metazachlor	.	29.08	54.63	604.04	.	.	74.87	3,568.54	5,538.65	688.75	.	.	10,558.57
Metconazole	.	.	.	42.23	29.99	230.17	.	.	.	.	.	.	302.39
Methiocarb	.	.	.	662.94	263.02	.	.	.	.	.	.	.	925.97
Methoxyfenozide	.	.	.	.	< 0.01	.	7.18	.	.	.	.	.	7.5
Methyl bromide	.	.	.	.	10,652.77	14,632.45	.	.	.	.	.	.	25,285.22
Metrafenone	.	.	19.26	.	.	.	.	.	.	.	.	.	19.26
Metsulfuron-methyl	.	< 0.01	11	17.06	54.57	.	.	.	.	.	< 0.01	1.7	84.82
Myclobutanil	.	.	< 0.01	11.96	12.81	16.08	24.17	2.73	.	.	.	.	67.93
Napropamide	.	236.84	.	.	.	.	.	427.1	163.18	.	.	.	827.12
Nicosulfuron	.	.	.	.	1.86	< 0.01	.	.	.	.	.	.	2.68
Nicotine	.	.	.	.	.	1,112.47	.	.	.	.	.	.	1,112.47
Oxadiazon	28.85	30.82	186.36	68.72	.	.	.	116.07	< 0.01	.	.	.	430.84
Oxadixyl	.	.	6.8	6.88	.	.	.	.	3.18	.	.	.	16.86
Oxycarboxin	.	.	< 0.01	.	.	.	.	.	.	.	.	.	0.06
Paclobutrazol	.	.	.	.	10.97	27.88	4.41	.	.	.	.	.	43.25
Paraquat	11.7	113.48	25.41	39.4	39.19	95.4	3.47	< 0.01	24.87	1.94	.	< 0.01	355.82
Penconazole	.	.	.	< 0.01	< 0.01	< 0.01	.	.	.	.	.	.	2.47
Pendimethalin	55.67	1,513.31	1,325.99	1,466.80	583.04	.	.	1.91	2,564.58	6,777.54	25,129.67	7,804.44	47,222.94
Permethrin	.	< 0.01	.	.	.	.	.	.	.	.	.	.	0.23
Phenmedipham	.	.	.	6.86	.	.	.	.	.	26.14	.	.	33
Picloram	.	.	11.07	.	.	.	.	.	.	.	.	.	11.07
Picolinafen	.	.	.	.	.	.	.	.	.	74.17	103.1	.	177.27
Picoxystrobin	.	.	.	692.09	832.28	330.05	.	.	.	.	.	.	1,854.42
Pirimicarb	< 0.01	1.86	< 0.01	< 0.01	19.31	489.4	139.79	3.91	3.91	2.94	.	.	662.97
Pirimiphos-methyl	.	.	.	.	.	.	4.17	.	< 0.01	.	.	.	4.43
Plant extracts	.	.	.	.	< 0.01	1.37	.	.	.	.	.	.	1.55
Prochloraz	6.06	4.74	3.71	< 0.01	< 0.01	346.79	1.34	< 0.01	< 0.01	1.69	3.03	4.87	373.78
Prohexadione-calcium	.	.	.	.	.	2.06	1.38	.	.	.	.	.	3.44
Propachlor	.	.	.	201.81	14.67	617.74	535.81	19.06	61.75	3.18	.	.	1,454.03

Active substance	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Annual
Propamocarb hydrochloride	.	19.19	52.1	.	.	.	.	.	7.31	.	.	.	78.6
Propaquizafop	.	67.7	81.41	166.46	75.54	10.9	.	.	644.67	25.25	230.29	2.68	1,304.91
Propiconazole	.	.	193.46	29.17	.	134.78	.	.	.	.	.	.	357.41
Propoxycarbazone-Na	.	.	41.94	60.15	10.68	.	.	.	.	.	.	.	112.77
Propyzamide	1.2	13.96	.	.	31.66	.	.	.	.	.	78.76	.	125.58
Prosulfuron	.	.	.	.	1.11	23.54	.	.	.	.	.	.	24.65
Prothioconazole	.	.	.	.	6.31	.	.	.	.	.	.	.	6.31
Pymetrozine	4.47	.	6.56	11.33	9.84	8.94	< 0.01	8.64	.	.	.	.	50.37
Pyraclostrobin	.	.	60.27	109.93	1,094.31	38.7	.	.	.	.	.	.	1,303.21
Pyrifenox	.	.	.	.	< 0.01	< 0.01	.	< 0.01	.	.	.	.	0.33
Pyrimethanil	.	.	.	12.85	6.53	42.79	.	.	.	.	.	.	62.18
Quinmerac	.	.	.	.	.	.	.	1,030.09	333.14	89.67	.	.	1,452.90
Quinoclamin	.	.	28.27	.	.	28.27	.	.	.	.	.	.	54.37
Quinoxyfen	.	.	319.52	12.72	182.87	150.33	.	.	.	.	.	.	665.44
Rimsulfuron	.	.	.	.	.	2.88	.	.	.	.	.	.	2.88
Silthiofam	.	.	.	.	.	.	.	.	58.1	76.94	.	.	135.04
Simazine	6.54	175.96	2,683.48	16.51	51.65	.	.	.	3.69	468.4	2,198.89	< 0.01	5,605.95
Spinosad	.	.	.	.	.	1.9	5.31	< 0.01	.	.	.	.	7.59
Spiroxamine	.	.	.	68.74	65.43	32.36	.	.	.	.	.	.	166.53
Sulfosulfuron	.	.	16.42	34.01	1.71	.	.	.	.	.	.	.	52.13
Sulphur	.	.	796.5	570.93	16.61	69.51	50.12	227.87	178.37	186.13	111.68	.	2,207.72
Tar oil	.	2,106.53	.	.	.	.	.	.	.	.	.	.	2,106.53
Tau-fluvalinate	.	.	.	86.47	3.4	198.73	.	.	20.94	34.76	65.32	1.08	410.69
Tebuconazole	.	13.96	30.55	692.84	186.08	2,595.07	.	.	5.25	37.69	4.39	.	3,565.82
Tebufenpyrad	.	.	.	< 0.01	< 0.01	< 0.01	2.05	2.34	.	.	.	.	5.14
Teflubenzuron	.	.	.	1.32	1.1	< 0.01	1.4	< 0.01	.	.	.	.	4.49
Tefluthrin	.	.	.	.	.	.	.	.	.	.	.	.	2.87
Tepraloxydim	.	.	.	< 0.01	55.81	.	.	.	.	107.6	138.5	.	302.67
Terbuthylazine	.	.	111.47	.	.	.	.	.	.	.	.	.	111.47
Terbutryn	.	.	259.84	.	.	.	.	.	.	.	.	.	259.84
Tetradifon	.	.	.	.	.	.	< 0.01	.	.	.	.	.	0.65
Thiacloprid	.	.	.	5.44	< 0.01	.	1.31	.	< 0.01	< 0.01	.	.	8.01

Active substance	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Annual
Thifensulfuron-methyl	.	17.05	.	1.89	155.87	.	.	.	.	13.31	48.04	.	236.17
Thiophanate-methyl	.	.	.	.	1,474.19	7.01	7.01	.	7.01	.	.	.	1,495.23
Thiram	.	.	30.74	128.48	16.28	3.14	2.46	87.44	219.88	178.74	65.22	.	732.37
Tolclofos-methyl	.	.	.	.	.	2.51	101.62	.	.	6.74	.	.	111.03
Tolylfluanid	.	.	.	.	5.73	11.46	4.52	7.64	.	.	.	.	29.36
Tralkoxydim	.	.	.	867.63	2,571.47	273.15	.	.	.	.	.	.	3,712.25
Triadimenol	.	.	.	.	.	.	.	.	153.71	501.2	.	.	654.9
Tri-allate	.	.	.	.	.	.	.	.	.	2,240.45	.	.	2,240.45
Triazamate	.	.	.	11.4	.	15.29	.	.	.	.	.	.	26.69
Triazoxide	.	18.98	41.53	1.49	.	.	.	.	7.13	49.59	.	.	118.73
Tribenuron-methyl	.	.	43.36	.	5.19	.	.	.	< 0.01	.	6.63	.	55.49
Triclopyr	.	.	.	171.72	15.54	.	.	.	.	.	.	.	187.26
Trifloxystrobin	.	.	.	187.65	723.14	103.75	.	.	.	.	.	2.5	1,017.04
Trifluralin	.	1,038.19	974	1,205.51	.	.	.	5,360.58	1,796.20	850.09	1,779.10	2,206.10	15,209.77
Triforine	< 0.01	1.96	.	< 0.01	< 0.01	3.22	1.12	< 0.01	< 0.01	< 0.01	.	.	7.37
Trinexapac-ethyl	.	.	.	653.92	122.78	.	.	.	.	.	.	.	776.7
Triticonazole	.	.	.	.	.	.	.	.	.	3.17	.	.	3.17
Zeta-cypermethrin	.	.	.	.	.	4.57	.	.	.	.	.	.	4.57

Appendix 2. Supply water analysis

National Laboratory Service

Analytical Report

Final Report

Report ID - 20052385 - 1

Batch description: Aqueous Samples

UKAS TESTING 0754

Reported on: 22-May-2013

Lucia Privitera
CEFAS
Pakefield Road
Lowestoft
Suffolk
NR33 0HT

Dear Lucia

Please find attached the results for the batch of 3 samples described below.

Samples Registered on:	13-May-2013
Analysis Started on:	14-May-2013
Analysis Completed on:	22-May-2013
Results for Batch Number	20052385
Your Purchase Order Number:	20018857

You will be invoiced shortly by our accounts department.

If we can be of further assistance then please do not hesitate to contact us.

Yours sincerely

William Fardon
Customer Services Team Manager
Tel: (0113) 231 2177
nls@environment-agency.gov.uk

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Page 1 of 5

National Laboratory Service

Analytical Report

Final Report

Report ID - 20052385 - 1

Batch description: Aqueous Samples

0754

Reported on: 22-May-2013

Client: CEFAS

Folder No: 002370496
Comments: Sea Water
Quote No: 10041

Project: Aqueous analysis
Sample Point Name: CC CEFAS
Sampled on: 10-May-13 @ 10:00
Matrix: Saline Water

Analyte	Result	Units	Flag	MRV	Accred	Lab ID	Testcode
Arsenic	1.18	ug/l		1	UKAS	SX	30
Selenium	<1	ug/l		1	UKAS	SX	39
Aluminium	<40	ug/l		40	None	SX	321
Antimony	<10	ug/l		10	UKAS	SX	321
Beryllium	<10	ug/l		10	UKAS	SX	321
Cobalt	<10	ug/l		10	UKAS	SX	321
Molybdenum	<30	ug/l		30	UKAS	SX	321
Tin	<20	ug/l		20	UKAS	SX	321
Titanium	<20	ug/l		20	UKAS	SX	321
Vanadium	<20	ug/l		20	UKAS	SX	321
Cadmium	<0.04	ug/l		0.04	UKAS	SX	89
Copper	1.06	ug/l		0.2	UKAS	SX	89
Lead	0.114	ug/l		0.04	UKAS	SX	89
Nickel	0.571	ug/l		0.3	UKAS	SX	89
Zinc	1.08	ug/l		0.4	UKAS	SX	89
Barium	<100	ug/l	∞	100	UKAS	SX	95
Boron	4420	ug/l	∞	700	UKAS	SX	95
Calcium	383	mg/l	∞	10	UKAS	SX	95
Iron	<100	ug/l	∞	100	UKAS	SX	95
Magnesium	1090	mg/l	∞	3	UKAS	SX	95
Manganese	<20	ug/l	∞	20	UKAS	SX	95
Potassium	333	mg/l	∞	1	UKAS	SX	95
Sodium	9150	mg/l	∞	20	UKAS	SX	95
Strontium	6940	ug/l	∞	200	UKAS	SX	95
Sulphate as SO4	2290	mg/l	∞	5	UKAS	SX	95
Mercury	<0.01	ug/l		0.01	UKAS	SX	247
Chromium	<0.5	ug/l		0.5	UKAS	SX	92

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National Laboratory Service

Analytical Report

Final Report

Report ID - 20052385 - 1

Batch description: Aqueous Samples

UKAS 0754

Reported on: 22-May-2013

Client: CEFAS

Folder No: 002370499
Comments: Tap Water
Quote No: 10041

Project: Aqueous analysis
Sample Point Name: CC CEFAS
Sampled on: 10-May-13 @ 10:00
Matrix: Potable waters

Analyte	Result	Units	Flag	MRV	Accred	Lab ID	Testcode
Arsenic	<1	ug/l		1	None	SX	30
Selenium	<1	ug/l		1	None	SX	39
Antimony	<1	ug/l		1	None	SX	36
Beryllium	<1	ug/l		1	None	SX	36
Cobalt	<1	ug/l		1	None	SX	36
Molybdenum	<3	ug/l		3	None	SX	36
Silver	<1	ug/l		1	None	SX	36
Tin	<2	ug/l		2	None	SX	36
Titanium	<2	ug/l		2	None	SX	36
Vanadium	<2	ug/l		2	None	SX	36
Aluminium	<10	ug/l		10	None	SX	34
Cadmium	0.415	ug/l		0.1	None	SX	34
Chromium	<0.5	ug/l		0.5	None	SX	34
Copper	2590	ug/l		1	None	SX	34
Lead	5.36	ug/l		2	None	SX	34
Nickel	4.75	ug/l		1	None	SX	34
Zinc	1140	ug/l		5	None	SX	34
Barium	46.4	ug/l		10	None	SX	38
Boron	<100	ug/l		100	None	SX	38
Calcium	153	mg/l		1	None	SX	38
Iron	<30	ug/l		30	None	SX	38
Lithium	<100	ug/l		100	None	SX	38
Magnesium	11.2	mg/l		0.3	None	SX	38
Manganese	<10	ug/l		10	None	SX	38
Potassium	6.44	mg/l		0.1	None	SX	38
Sodium	40.1	mg/l		2	None	SX	38
Strontium	1420	ug/l		20	None	SX	38
Sulphate as SO4	153	mg/l		10	None	SX	38
Mercury	<0.01	ug/l		0.01	None	SX	247

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National Laboratory Service

Analytical Report

Final Report

Report ID - 20052385 - 1

Batch description: Aqueous Samples

0754

Reported on:
22-May-2013

Client: CEFAS

Folder No: 002370500
Comments: Dechlorinated Tap Water
Quote No: 10041

Project: Aqueous analysis
Sample Point Name: CC CEFAS
Sampled on: 10-May-13 @ 10:00
Matrix: Potable waters

Analyte	Result	Units	Flag	MRV	Accred	Lab ID	Testcode
Arsenic	<1	ug/l		1	None	SX	30
Selenium	<1	ug/l		1	None	SX	39
Antimony	<1	ug/l		1	None	SX	36
Beryllium	<1	ug/l		1	None	SX	36
Cobalt	<1	ug/l		1	None	SX	36
Molybdenum	<3	ug/l		3	None	SX	36
Silver	<1	ug/l		1	None	SX	36
Tin	<2	ug/l		2	None	SX	36
Titanium	2.77	ug/l		2	None	SX	36
Vanadium	<2	ug/l		2	None	SX	36
Aluminium	<10	ug/l		10	None	SX	34
Cadmium	<0.1	ug/l		0.1	None	SX	34
Chromium	<0.5	ug/l		0.5	None	SX	34
Copper	10.5	ug/l		1	None	SX	34
Lead	<2	ug/l		2	None	SX	34
Nickel	1.74	ug/l		1	None	SX	34
Zinc	10.3	ug/l		5	None	SX	34
Barium	44.0	ug/l		10	None	SX	38
Boron	<100	ug/l		100	None	SX	38
Calcium	153	mg/l		1	None	SX	38
Iron	<30	ug/l		30	None	SX	38
Lithium	<100	ug/l		100	None	SX	38
Magnesium	12.4	mg/l		0.3	None	SX	38
Manganese	<10	ug/l		10	None	SX	38
Potassium	7.39	mg/l		0.1	None	SX	38
Sodium	62.1	mg/l		2	None	SX	38
Strontium	1420	ug/l		20	None	SX	38
Sulphate as SO4	171	mg/l		10	None	SX	38
Mercury	<0.01	ug/l		0.01	None	SX	247

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Analytical Report

Final Report

Report ID - 20052385 - 1

Batch description: Aqueous Samples

UKAS 0754

Reported on: 22-May-2013

Method Description Summary for all samples in batch Number 20052385

30	SX M Hydride As - Arsenic - acid digested; determined by HG-AAS
34	SX M ICPMS Routine - Metals - acid digested; determined by ICPMS
36	SX M ICPMS NR - Metals - acid digested; determined by ICPMS
38	SX M ICPOES Routine - Metals - acid digested; determined by ICPOES
39	SX M Hydride Se - Selenium - acid digested; determined by HG-AAS
89	SX M ICPMS Saline - Metals - saline matrix elimination; determined by ICPMS
92	SX M Misc Cr - Chromium - acid digested; determined by GF-AAS
95	SX M ICPOES Saline - Metals; Sulphate - acid digested; determined by ICPOES
247	SX M Mercury - determined by CV-AFS
321	SX M ICPMS NR Saline - Metals - acid digested; determined by ICPMS

Simon Padley
Laboratory Site Manager

All reporting limits quoted are those achievable for clean samples of the relevant matrix. No allowance is made for instances when dilutions are necessary owing to the nature of the sample or insufficient volume of the sample being available. In these cases higher reporting limits may be quoted and will be above the MRV.

Solid sample results are determined on a "dried" sample fraction except for parameters where the method description identifies that "as received" sample was used.

Key to Results Flags:

DC Analysis started outside of specified holding time. It is possible that the results may be compromised.

Please note all samples will be retained for 10 working days for aqueous samples and 30 working days for solid samples after reporting unless otherwise agreed with Customer Services

Key to Accreditation: UKAS = Methodology accredited to ISO/IEC 17025:2005, MCerts = Methodology accredited to MCerts Performance Standard for testing of soils, none = Methodology not accredited

Key to Lab ID: LE = Leeds, LL = Llanelli, NM = Nottingham, SX = Starcross, SC = Sub-Contracted outside NLS, FI = Field Data, NLS = Calculated

Any subsequent version of this report denoted with a higher version number will supersede this and any previous versions

END OF TEST REPORT

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Appendix 3. Eel Health Report

Lucia Privitera

Salmon and Freshwater Fisheries Group

Cefas

Pakefield Road

Lowestoft

Suffolk

NR33 0HT

Our ref:RIV00790A/20004

17 December 2009

Dear Lucia

Health screening of Eels taken from STOUR RIVER CATCHMENT

This is to confirm the results of the health screen from the eels sampled from the trap in the Stour River Catchment on Tuesday, 17 November 2009 and that all testing has been completed.

From our examination of the samples we can report the following results

Visual examination

Externally several of the eels provided had lesions around the caudal peduncle and tail fin. The fish otherwise appeared to be in good health. Weights of the five eels were 273, 342, 431, 494 and 482 grammes. Internally, the only observation was that some fish had fatty deposits surrounding the heart.

Parasitology

The following parasites were noted

- *Anguillicoloides crassus* (Nematoda) in swim bladder

- *Paraquimperia tennerima* (Nematoda) in intestine
- *Acanthocephalus lucii* (Acanthocephala) in intestine
- *Pseudodactylogyrus sp.* (Monogenea) in gills

Bacteriology

Swabs were taken from the kidney for each of the 5 fish and plated onto TSA. From fish number 4 *Aeromonas hydrophila* was cultured and identified. No significant organisms were cultured from the other fish.

Virology

Samples of spleen, kidney and brain were taken from each fish and pooled. This was then inoculated onto the following cell lines BF-EP, EP-20, CH-15, FH-15 and FH-20's. These have all proven negative for serious viruses e.g. VHS (viral haemorrhagic septicaemia), IHN (infectious haematopoietic necrosis), IPN (infectious pancreatic necrosis), SVC (spring viraemia of carp) and eel rhabdovirus. A PCR for eel herpesvirus was also negative.

Histopathology

Fish 1. Liver, spleen and pronephros had occasional macrophage aggregates (MAs) (within normal limits for eels). The heart had some small foci of inflammation. Intestine and skin – showed no apparent disease (NAD). Gills with small *Myxidium giardi* (MG) cysts, not regarded as significant.

Fish 2. Spleen with large MG cyst and several small cysts in the gills, no significant host reaction. Other tissues NAD.

Fish 3. Heart, spleen, pronephros and liver - NAD. Eroded skin epithelium, likely to be trauma. Gill with secondary lamellar fusion with no MG cysts.

Fish 4. Few MG cysts in the gill. Skin epithelium erosion. Other tissues NAD.

Fish 5. MA's as in fish 1. Focal mucous cell proliferation in secondary gill lamella. Other organs NAD.

Note - for all fish kidney (mesonephros) was not submitted for examination (S.W. Feist)

We are pleased to report that the visual examination of representative stocks on your site and post mortem examinations of the stocks sampled provided no evidence for the presence of any notifiable disease. However, the parasite *Anguillicoloides crassus* is classified as a category 2 parasite by the Environment agency.

It is thought likely that the lesions on the fish were most likely caused by the traps or holding units before collection. Despite their reputation for being hardy, eels can be surprising sensitive to handling stress so all efforts should be taken to minimise this.

If you require further clarification on any of the above please do not hesitate to contact the laboratory.

Yours faithfully

Keith Jeffery

Fish Health Inspector

Appendix 4. Dissemination

Peer reviewed publications

Privitera, L., Aarestrup, K. & Moore, A. 2014. Impact of a short-term exposure to tributyl phosphate on morphology, physiology and migratory behaviour of European eels during the transition from freshwater to the marine environment. Ecology of Freshwater Fish 23(2): 171-180.

Privitera, L. & Moore, A. Mixtures of pesticides and their impact on silver eel migratory behaviour. (In prep).

Conference abstract

Privitera, L., Assunçao, M. & Moore, A. Effects of a flame retardant on the adaptation of glass eels to freshwater. Institute of Fisheries Management, Annual Conference. 19-21 October 2010, Portsmouth, United Kingdom. (Poster presentation)

Privitera, L., Aarestrup, K. & Moore, A. Impact of a short-term exposure to tributyl phosphate on morphology, physiology and migratory behaviour of European eels during the transition from freshwater to the marine environment. Ecology and Conservation of Freshwater Fish. 28 May- 02 June 2012, Vila Nova de Cerveira, Portugal. (Oral communication)

Privitera, L., Bean, T., Lyons, B. & Moore, A. Effects of short term metal exposure on glass eel DNA integrity and freshwater adaptation. Eel Genome Symposium. 16-17 January 2014, Leiden, The Netherlands. (Oral communication)

Privitera, L. & Moore, A. Effects of silver eel exposure to a pesticide mixture on their physiology and downstream migration. Institute of Fisheries Management, Tagging and telemetry workshop, 22-23 July 2014, Leeds, United Kingdom. (Oral communication).

Appendix 5. Ethical documents

Centre for Environment
Fisheries & Aquaculture
Science

Cefas

Lowestoft

24th September 2015

The effects of diffuse pollution on European eel – Lucia Privitera.

Thesis submitted in partial fulfilment for the award of degree of Doctor of Philosophy at the University of Portsmouth.

The experimental work described in the above Thesis was undertaken at the Centre for Environment, Fisheries and Aquaculture Science, Lowestoft Laboratory. Cefas affirms the importance of animal welfare and commits to meet in full the obligations under the Animals (Scientific Procedures) Act 1986.

The experiments were carried out under a Home Office Animals (Scientific Procedures) Act 1986 Personal Licence (PIL 80/10073) held by Lucia Privitera and under appropriate Project Licences held by Dr Andrew Moore at Cefas (PPL 80/2174 and 70/7588).

All experimental work was discussed and approved by the Animal Welfare and Ethical Review Body Statistician and prior to implementation approved by the Named Animal Care and Welfare Officer (NACWO).

Julian Metcalfe

Chair, Animal Welfare and Ethical Review Body, Lowestoft Laboratory
Centre for Environment, Fisheries & Aquaculture Science.

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FORM UPR16

Research Ethics Review Checklist

University of Portsmouth

Please include this completed form as an appendix to your thesis (see the Postgraduate Research Student Handbook for more information

Postgraduate Research Student (PGRS) Information				**Student ID:**	475401		
PGRS Name:	Lucia Privitera						
Department:	Biological Science	**First Supervisor:**	Dr Trevor Willis				
Start Date: (or progression date for Prof Doc students)	**01 October 2009**						
Study Mode and Route:	Part-time	☒	MPhil	☐	MD	☐	
	Full-time	☐	PhD	☒	Professional Doctorate	☐	

Title of Thesis:	The effects of diffuse pollution on European eel
Thesis Word Count: (excluding ancillary data)	44123

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist:
(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: http://www.ukrio.org/what-we-do/code-of-practice-for-research/)

a)	Have all of your research and findings been reported accurately, honestly and within a reasonable time frame?	YES ☒ NO ☐
b)	Have all contributions to knowledge been acknowledged?	YES ☒ NO ☐
c)	Have you complied with all agreements relating to intellectual property, publication and authorship?	YES ☒ NO ☐
d)	Has your research data been retained in a secure and accessible form and will it remain so for the required duration?	YES ☒ NO ☐
e)	Does your research comply with all legal, ethical, and contractual requirements?	YES ☒ NO ☐

Candidate Statement:

I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)

Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):	N/A

If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain below why this is so:

Work carried out while employed at Cefas, Lowestoft, under Cefas Home Office Licence

Signed *(PGRS)*: Lucia Privitera **Date:** 23 September 15

UPR16 – August 2015